TECHNICAL REPORT
RERF TR 17-88
業 績 報 告 書

DETECTION OF A CIRCULATING TUMOR-ASSOCIATED ANTIGEN WITH A MURINE MONOCLONAL ANTIBODY, LISA 101, SELECTED BY REVERSED INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY

逆間接酵素抗体法で選出した マウスモノクローナル抗体 LISA 101 による循環腫瘍関連抗原の検出

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ACKNOWLEDGMENT

謝　辞

The authors gratefully acknowledge the excellent technical assistance of Ms. Kyoko Ozaki, Ms. Yoshiko Watanabe, and Ms. Tomoko Hokao of RERF. The assistance of Ms. Michiko Takagi and Ms. Mitsue Wakasa in typing the manuscript is greatly appreciated. The authors also express their thanks to the following collaborators for providing sera: Dr. Hideo Sasaki, National Kure Hospital; Dr. Noritomo Senoo and Dr. Teruomi Miyazawa, Hiroshima City Hospital; Dr. Masao Kuwabara, Hiroshima Prefectural Hospital; Dr. Hiroya Egawa, Asa City Hospital; Dr. Mikio Fujita, Yoshijima Hospital; Dr. Kenichiro Sadamoto, National Sanatorium Hiroshima Hospital; Dr. Shiro Nakai, Hiroshima Memorial Hospital; the staff of the Second Department of Surgery and the First Department of Internal Medicine, Hiroshima University School of Medicine. Dr. Reiji Kannagi, Department of Clinical Laboratory, Kyoto University School of Medicine, is acknowledged for providing monoclonal antibody FH 6; and Dr. Stuart C. Finch, Chief of Research, RERF for editing the manuscript.

多大なる技術的援助をいただいた放影研の尾崎恭子，渡辺芳子，外尾智子各氏，並びに原稿をタイプされた高木迪子，若狭光枝の両氏に深く謝意を表する．血清を提供いただいた国立呉病院の佐々木英夫博士，広島市民病院の妹尾紀具博士，宮沢輝臣博士，広島県立病院の桑原正雄博士，安佐市民病院の江川博彌博士，吉島病院の藤田幹夫博士，国立療養所広島病院の定本謙一郎博士，広島記念病院の中井志郎博士及び広島大学医学部第２外科学教室並びに第１内科学教室のスタッフに対して，また，モノクローナル抗体 FH 6 を提供していただいた京都大学医学部検査部の神奈木玲児博士及び原稿を校閲していただいた放影研研究担当理事 Dr. Stuart C. Finch に対しても謝意を表する．

A paper based on this report was accepted for publication by:

本報告に基づく論文は下記の雑誌に受理された．

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The Radiation Effects Research Foundation (formerly ABCC) was established in April 1975 as a private nonprofit Japanese Foundation, supported equally by the Government of Japan through the Ministry of Health and Welfare, and the Government of the United States through the National Academy of Sciences under contract with the Department of Energy.

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Research Project 研究課題 16-81

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SUMMARY

In the presence of a characterized monoclonal antibody recognizing a soluble molecule, additional monoclonal antibodies reactive with unknown antigenic determinants on the molecule can be easily selected by reversed indirect enzyme-linked immunosorbent assay (RI-ELISA). A novel murine monoclonal antibody LISA 101 was selected by RI-ELISA against soluble antigens, which exist in sera and in pleural effusions derived from lung adenocarcinoma patients and which bear determinants recognized by the previously characterized murine monoclonal antibody KL-6. Antigenic determinants recognized by the LISA 101 antibody appear to be sialylated carbohydrate in nature and different from those recognized by previously reported monoclonal antibodies against sialylated carbohydrates, such as NS 19-9, FH 6, KL-6, C 50, CSLEX-1, and KM-93, suggested by competitive inhibition assay and immunostaining of tissues. A circulating antigen LISA 1-6 was detected by a bimonoclonal bideterminant assay using immobilized LISA 101 antibody

要 約

可溶性分子を認識する特異性既知のモノクローナル抗体があれば，その分子上に存在する未知の抗原決定基と反応する他の各種モノクローナル抗体が，逆間接酵素抗体法（RI-ELISA）によって容易に選択できる．肺腺癌患者から得られた血清や胸水中に検出され，かつ以前に樹立したマウスモノクローナル抗体 KL-6 によって認識される決定基をもつ可溶性抗原と反応する新しいマウスモノクローナル抗体 LISA 101 を RI-ELISA で選択した．競合阻止試験（competitive inhibition assay）や組織の免疫染色法から判定すると，LISA 101 抗体によって認識される各抗原決定基の性質は，シアル酸化糖鎖化合物であるが，同じようにシアル酸化糖鎖化合物と反応する NS 19-9，FH 6，KL-6，C 50，CSLEX-1 及び KM-93 などのモノクローナル抗体によって認識される抗原とは異なるようである．固相化 LISA 101 抗体と

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Approved 承認 25 August 1988

Printed 印刷 July 1989

and enzyme-labeled KL-6 antibody. It was found that serum LISA 1-6 levels were elevated in 63% (25/40) of patients with lung adenocarcinoma and in 92% (11/12) of patients with pancreatic carcinoma, but only in 6.5% (2/31) of patients with benign lung diseases and in 7.1% (1/14) of patients with pancreatitis.

The present observations indicate that the LISA 1-6 antigen may serve as a new tumor marker for adenocarcinomas of the lung and the pancreas. Additionally, the RI-ELISA may be a widely applicable method for selecting new monoclonal antibodies against as yet unknown antigenic determinants on soluble molecules.

INTRODUCTION

Similar to other biological molecules, tumor-associated mucins have been shown to possess multiple epitopes on their molecules.[1] In our previous study,[2] we showed that the murine monoclonal antibody KL-6 recognizes sialylated carbohydrate of the mucin and that the antigen level appeared to increase in sera from patients having a malignant disease, such as lung adenocarcinoma or pancreatic and breast cancers. We suspected that the mucin recognized by KL-6 antibody might also bear other different epitopes, and so we started to screen a new series of monoclonal antibodies which can be used in conjugation with KL-6 antibody by means of the indirect-ELISA method. For this purpose, we employed one modification which uses goat antimouse immunoglobulin antibodies as the primary immobilized antibody, termed "reversed indirect enzyme-linked immunosorbent assay (RI-ELISA)[3]." This RI-ELISA was shown to increase the antigen specificity somewhat as compared to the ordinary indirect-ELISA.[3]

Current techniques to develop monoclonal antibodies reactive to circulating tumor antigens consist of screening hybridoma supernatants which are specifically reactive to tumor cells as a first step and later selecting from among these the ones that are reactive to the sera of cancer patients. In contrast, RI-ELISA uses sera or fluid from cancer patients directly at the time of selecting hybridoma supernatants. Thus, RI-ELISA is quite effective and superior to ordinary methods for screening monoclonal antibodies that are specific to circulating tumor antigens. This report describes the

酵素標識 KL-6 抗体を用いた bimonoclonal bideterminant assay によって流血中抗原 LISA 1-6 を検出した．血清中の LISA 1-6 抗原値は肺腺癌例の63％（40例中25例），膵臓癌例の92％（12例中11例）に増加が認められ，他方，良性肺疾患例ではわずか6.5％（31例中2例），膵臓炎例では7.1％（14例中1例）に増加が認められたにすぎない．

今回の観察結果は，LISA 1-6 抗原が肺及び膵臓の腺癌の新しい腫瘍マーカーになり得ることを示す．なお，RI-ELISA は，可溶性分子上の未知の抗原決定基と反応する新しいモノクローナル抗体を選別するために広く応用可能な方法であろう．

緒　言

他の生物学的分子と同様に腫瘍関連ムチンは分子上に多数のエピトープをもっていることが証明されている．[1] 我々の以前の研究[2]において，マウスモノクローナル抗体 KL-6 がムチンのシアル酸化炭水化物を認識することと，肺腺癌又は膵臓癌及び乳癌などの悪性疾患の罹患患者の血清中において，その抗原値は増加するようであることを示した．KL-6 抗体により認識されるムチンも他の異なるエピトープをもつことが予測されたので，間接 ELISA 法によって KL-6 抗体と共に使用できる新しい各種モノクローナル抗体の選別を開始した．この目的のために，我々は第一固相抗体としてヤギの抗マウス免疫グロブリン抗体を用いる「逆間接酵素抗体法（RI-ELISA）[3]」と呼ばれる改良法を用いた．この RI-ELISA 法は通常の間接 ELISA 法[3]と比べて抗原の特異性を若干増大させることが判明した．

血中腫瘍抗原に反応するモノクローナル抗体を産生する現行の技法では，第一段階として腫瘍細胞に特異的に反応するハイブリドーマ上清を選別し，次にこれらの中から癌患者の血清に反応するものを選択する．対照的に RI-ELISA 法ではハイブリドーマ上澄の選択時に癌患者の血清又は体液を直接使用する．したがって，RI-ELISA 法は血中腫瘍抗原に特異的なモノクローナル抗体の選別には極めて効果的で，通常の方法よりも優れている．本報では，KL-6 抗

results of screening for a new monoclonal antibody (LISA 101) using RI-ELISA in conjunction with KL-6 antibody.

MATERIALS AND METHODS

Reversed Indirect Enzyme-Linked Immunosorbent Assay. RI-ELISA was performed according to the procedures described previously.[3] In brief, each well of a 96-well microtest plate (Costar, Cambridge, Mass) for enzyme immunoassay (EIA) was sensitized overnight at 4°C with 100 μl of 10 μg/ml goat antimouse immunoglobulin antibodies (Cappel Laboratories, Cochramville, Penn). After successive washing with buffer A (0.05% Tween 20, 0.1% BSA and PBS; 0.01 M phosphate, 0.14 M NaCl, pH 7.4), 100 μl of hybridoma culture supernatant was added and incubated for one hour at room temperature. Then, 100 μl of an antigen solution diluted fivefold with mouse serum buffer (1% normal mouse serum, 10% FCS, PBS) was added and incubated for one hour. The wells were washed, followed by addition of 100 μl of horseradish peroxidase (HRP)-labeled KL-6 (HRP-KL-6) antibody diluted 100-fold with mouse serum buffer and incubated for one hour. After washing, 100 μl of OPDA solution (0.3% *o*-phenylenediamine dihydrochloride, 0.02% H_2O_2, 0.15 M citrate-phosphate buffer, pH 4.9) was added and was allowed to react for 30 minutes, after which the reaction was stopped by adding 100 μl of 1 N H_2SO_4. Then absorbance (OD_{492}) was determined.

Hybridoma Selection with RI-ELISA. Seventy-one culture supernatants from uncloned hybridomas were examined for their reactivities to the KL-6 antigen by means of RI-ELISA. The hybridomas were produced by fusing NS-1 cells and splenocytes from mice immunized with pulmonary adenocarcinoma cell line VMRC-LCR.[2] KL-6 monoclonal antibody, recognizing mucinlike glycoprotein, had been established from one of these hybridomas, as described previously.[2] Two sets of specimens were used as antigens: pleural effusions which were pooled from 15 lung adenocarcinoma patients or from 10 patients who had pulmonary tuberculosis, and sera obtained from either lung adenocarcinoma patients (pooled from 20 patients at stages III and IV) or from healthy persons (pooled from 30 individuals). Antigen levels, measured with the sandwich-type ELISA technique, usign KL-6 antibody[2] in these specimens, were 3,450, 270, 1,300, and 390 U/ml, respectively. A culture

体と共に RI-ELISA 法を用いて新モノクローナル抗体（LISA 101）を選別した結果を示す．

試料及び方法

逆間接酵素抗体法． 既報[3]で記述した手順に従って RI-ELISA 法を実施した．簡単に述べると，酵素免疫測定（EIA）用の96穴のマイクロテストプレート（Costar, Massachusetts 州 Cambridge）の各穴を10 μg/ml のヤギの抗マウス免疫グロブリン抗体（Cappel Laboratories, Pennsylvania 州 Cochramville）100 μl を用いて4℃で一晩感作させた．緩衝液 A（0.05% Tween 20，0.1% BSA 及び PBS；0.01 M リン酸，0.14 M NaCl，pH 7.4）で何回も洗浄した後，ハイブリドーマ培養上澄100 μl を添加し，室温で1時間反応させた．次にマウス血清緩衝液（1%正常マウス血清，10% FCS，PBS）で5倍に希釈した抗原溶液100 μl を添加し，1時間反応させた．各穴を洗浄し，マウス血清緩衝液で100倍に希釈した horseradish ペルオキシダーゼ（HRP）標識 KL-6（HRP-KL-6）抗体100 μl を添加し1時間反応させた．洗浄後，100 μl の OPDA 溶液（0.3% *o*-phenylenediamine dihydrochloride，0.02% H_2O_2，0.15 M クエン酸リン酸緩衝液，pH 4.9）を添加し30分間反応させた後，1 N H_2SO_4 100 μl を添加し反応を停止させ，それから吸光度（OD_{492}）を測定した．

RI-ELISA 法によるハイブリドーマの選択． 未クローン化ハイブリドーマから得た71個の培養上澄の KL-6 抗原に対する反応性を RI-ELISA 法を用いて調べた．NS-1 細胞と肺腺癌細胞株 VMRC-LCR で免疫したマウスの脾細胞とを融合してハイブリドーマを産出した．[2] 既報[2]で記述したように，これらのハイブリドーマのうちの一つから，ムチン型糖蛋白質を認識する KL-6 モノクローナル抗体を確立した．抗原として2組の標本，すなわち肺腺癌患者15名又は肺結核患者10名からプールした胸水と，肺腺癌患者から得た血清（臨床病期Ⅲ及びⅣの患者20名からプール）又は健常人から得た血清（30名からプール）を使用した．KL-6 抗体[2]を使用し sandwich 型 ELISA 法で測定したこれらの標本の抗原値は，各々3,450，270，1,300 及び390 U/ml であった．IgG_1 抗体を

supernatant of MOPC-21 cells, which consists of mouse myeloma cells secreting IgG_1 antibody, was used as a negative control.

Determination of Cross-Reactivity of Monoclonal Antibodies. Newly selected monoclonal antibodies could possibly cross-react with the same antigenic determinant that is recognized by the KL-6 antibody. Competitive inhibition assays were performed to test this possibility. A standard solution of KL-6 antigen (256 U/ml)[2] was incubated with immobilized KL-6 antibody in wells of an EIA plate. After washing, several concentrations of newly selected monoclonal antibodies were added to each well, together with 100-fold diluted HRP-KL-6 antibody. The binding of HRP-KL-6 antibody was measured with OPDA as the percentage exhibiting binding as compared to the percentage of the control MOPC-21 (IgG_1) antibody.

Cross-reactivity of these monoclonal antibodies with NS 19-9[4,5] and FH 6[6,7] antibodies, which recognize sialylated Lewis^a antigen and sialylated Lewis^x i antigen, respectively, was examined using a kit of CA 19-9 (Fuji Rebio Inc., Tokyo, Japan) and SLX "Otsuka" (Otsuka Assay Institute, Tokushima, Japan), respectively.

Conjugation of Monoclonal Antibodies with HRP. KL-6 antibody was conjugated with HRP using the method of Nakane and Kawaoi[8] as described elsewhere.[7] On the other hand, one of the above-mentioned newly selected monoclonal antibodies, LISA 101, was conjugated with HRP using the method of Carlsson et al,[9] because this method induces increased sensitivity when compared with the method of Nakane and Kawaoi. Two different cross-linking agents are used in this method, one being N-(ε-maleimidocaproyloxy) succinimide (EMCS, Dojindo Lab., Kumamoto, Japan) and the other being N-succinimidyl 3-(2-pridyldithio) propionate (SPDP, Sigma Chemical Co., St. Louis, MO). Briefly, 4 mg of LISA 101 antibody was dissolved in 1.0 ml of phosphate-buffered saline (PBS, 0.1 M phosphate, 0.1 M NaCl, pH 7.5). After adding 0.1 ml of 0.02 m SPDP ethanol solution, the mixture was incubated for 30 minutes at room temperature, desalinated and equilibrated with acetate buffer (0.02 M acetate, 0.1 M NaCl, pH 4.5) by passing through a Sephadex G-25 column. To the solution was added 0.15 ml of 1 M dithiothreitol, followed by incubation for 30

分泌するマウス骨髄腫細胞から成るMOPC-21細胞の培養上清を陰性対照として用いた.

モノクローナル抗体の交差反応性の測定. 新たに選択されたモノクローナル抗体は, KL-6抗体により認識される同一の抗原決定基と交差反応する可能性がある. この可能性を検定するために競合阻止反応試験を行った. KL-6抗原の標準液(256 U/ml)[2]をEIAプレートの穴の固相KL-6抗体と反応させた. 洗浄後, 幾つかの濃度の新しく選択されたモノクローナル抗体を100倍に希釈したHRP-KL-6抗体と共に各穴に添加した. HRP-KL-6抗体の結合は, 結合を示す割合を対照MOPC-21(IgG_1)抗体の結合割合と比較してOPDAを用いて測定した.

これらの抗体と各々シアル酸化Lewis^{a}抗原及びシアル酸化Lewis^x i抗原を認識するNS 19-9抗体[4,5]及びFH 6抗体[6,7]との交差反応性をCA 19-9キット(Fuji Reibo, 東京)とSLX"Otsuka"キット(大塚アッセイ研究所, 徳島)とをそれぞれ用いて調べた.

モノクローナル抗体のHRPとの結合. 別報[7]の記述のとおりNakane及びKawaoi法[8]を用いてKL-6抗体をHRPと結合させた. 一方, Carlssorら[9]の方法を用いて上述の新しく選択されたモノクローナル抗体の一つLISA 101をHRPと結合させたのは, この方法がNakane及びKawaoiの方法と比べて感度の増加をもたらすからである. この方法では二つの異なる交差結合試薬, N-(ε-maleimidocaproyloxy)succinimide (EMCS, Dojindo Lab., 熊本)とN-succinimidyl 3-(2-pridyldithio)propionate (SPDP, Sigma Chemical Co., Missouri州St. Louis)を用いた. 簡単に言えば, LISA 101抗体4 mgをリン酸緩衝食塩水(PBS, 0.1Mリン酸, 0.1M NaCl, pH 7.5)1.0ml中で溶解した. 0.02M SPDPエタノール溶液0.1mlを添加した後に混合液を室温で30分間反応させ, Sephadex G-25カラム中を通過させて脱塩し酢酸緩衝液(0.02M酢酸, 0.1M NaCl, pH 4.5)で平衡化した. その溶液に1 M dithiothreitol 0.15mlを添加

minutes at room temperature. The solution was then desalinated and equilibrated with PBS containing 0.001 M ethylenediaminetetraacetic acid (EDTA) by passing through a Sephadex G-25 column. The effluent solution contained SPDP-conjugated LISA 101 antibodies. Simultaneously, 1.0 mg of HRP was dissolved in 0.15 ml of PBS. After adding 15 μl of 50 μmol EMCS in a N, N-dimethylformamide solution, the mixture was incubated for 30 minutes at 30°C. The solution was desalinated and equilibrated with PBS passing through a Sephadex G-25 column, and it was mixed with SPDP-conjugated LISA 101 antibodies. The mixture was incubated for 20 hours at 23°C, and the first peak obtained upon gel filtration in the Sephadex G-200 column was used as the HRP-LISA 101 antibody.

Quantification of Circulating Antigens LISA 1-6 and LISA 101. A soluble antigen, termed "LISA 1-6", is measured with the sandwich-type ELISA using immobilized LISA 101 antibody on polystyrene beads and HRP-KL-6 antibody. On the other hand, a soluble antigen, LISA 101, is detected by sandwich-type ELISA using LISA 101 antibody as both the immobilized antibody and HRP-labeled antibody.

Polystyrene beads were sensitized with purified LISA 101 antibodies as will be described. Polystyrene beads (6.4 mm, Wako, Osaka, Japan) were incubated at 4°C for 48 hours in a LISA 101 antibody solution (50 μg/ml LISA 101 antibody, 0.1 M $NaHCO_3$, pH 8.4) and were stored at 4°C for later use.

The antibody-sensitized beads were allowed to react overnight with 0.3 ml of the antigen specimen at 37°C in a plastic tube, after which they were washed three times with a saline solution. Thereafter, 0.3 ml of HRP-labeled antibodies diluted 100-fold with a buffer B (0.1% normal mouse serum, 10% FCS, PBS) was added and incubated for 3 hours. After washing, the beads were transferred to a polystyrene tube (Elkay Products Inc., boston, Mass) and 0.3 ml of OPDA solution was added and incubated for 30 minutes. Enzyme reaction was stopped by adding 1.0 ml of 2N hydrochloric acid and absorbance (OD_{492}) was measured.

For quantification of both the soluble antigens LISA 1-6 and LISA 101, a pleural effusion from a lung cancer patient was used as a standard reference sample in every assay. This reference pleural

し，室温で30分間反応させた．この溶液を Sephadex G-25カラム中を通過させて脱塩し，0.001 M ethylenediaminetetraacetic acid (EDTA) を含むリン酸緩衝食塩水で平衡化した．溶出液は SPDP 結合 LISA 101抗体を含んでいた．同時に，HRP 1.0 mg を PBS 0.15 ml中で溶解した．50 μmol の EMCS 15 μl を N, N-dimethylformamide 溶液中に添加した後で，混合液を30℃ で30分間反応させた．溶液を Sephadex G-25カラム中を通過させ脱塩し，PBS で平衡化し，その溶液を SPDP 結合 LISA 101抗体と混合した．混合液を23℃ で20時間反応させ，Sephadex G-200 カラムによるゲル濾過で最初に得られたピークを HRP-LISA 101抗体として使用した．

血中抗原 LISA 1-6 及び LISA 101 の定量化． ポリスチレン・ビーズ上の固相 LISA 101抗体と HRP-KL-6 抗体とを用いる sandwich 型 ELISA 法で"LISA 1-6"と呼ばれる可溶性抗原を測定した．一方，固相抗体かつ HRP 標識抗体として LISA 101抗体を用いる sandwich 型 ELISA 法で可溶性抗原 LISA 101を検出した．

ポリスチレン・ビーズを後述のように精製 LISA 101抗体で感作した．ポリスチレン・ビーズ（6.4 mm，和光，大阪）を LISA 101抗体溶液（50 μg/ml LISA 101抗体，0.1 M $NaHCO_3$，pH 8.4）中で48時間 4 ℃で反応させ，後で使用するために4 ℃で保存した．

抗体感作ビーズをプラスチック管の中で37℃ で抗原液0.3 ml と一晩反応させた後，食塩水で3 回洗浄した．その後，緩衝液 B（0.1%正常マウス血清，10% FCS，PBS）で100倍に希釈した HRP 標識抗体0.3 ml を添加し，3時間反応させた．洗浄後，ビーズをポリスチレン管（Elkay Products 社，Massachusetts 州 Boston）に移し，OPDA 溶液 0.3 ml を添加し30分間反応させた．2 N 塩酸1.0 ml を添加し酵素反応を停止させ，吸光度（OD_{492}）を測定した．

両可溶性抗原 LISA 1-6 及び LISA 101の定量化には，各測定ごとに同一の肺癌患者の胸水を標準基準サンプルとして用いた．この基準胸水は両抗原を

effusion had 16,000 U/ml of both antigens. Clinical specimens were treated with a buffered diluent, ninefold for LISA 1-6 and fivefold for LISA 101 antigen quantification.

16,000 U/ml 含んでいた．臨床検体は LISA 1-6 抗原の定量には9倍，LISA 101抗原の定量には5倍の希釈緩衝液で処理した．

Clinical Materials. Sera from 99 healthy controls were provided by Dr. K. Kiyosada, Nippon Telegraph and Telephone Corporation Health Care Center, Hiroshima, Japan. They reported no remarkable past clinical histories and showed no abnormalities in follow-up examinations, including analysis of peripheral blood cells, urinalysis, fecal examinations, serological tests, chest X ray, and upper gastrointestinal tract examination. Sera from patients with several types of malignancies and benign diseases were provided by several hospitals. These patients were diagnosed by pathological or other clinical examinations.

臨床試料．広島の日本電信電話公社健康管理センターの清貞和紀博士が健常対照者99名の血清を提供された．これらの対照者は過去に特記すべき既往歴を有さず，末梢血球数算定，尿検査，糞便検査，血清検査，胸部X線，上部胃腸管X線検査などの追跡検査で異常を示さなかった．幾つかの病院から数種類の悪性腫瘍及び良性疾患に罹患した患者の血清が提供された．これらの患者は病理検査又は他の臨床検査によって診断された．

Antigen Biochemical Studies

Test of sensitivity to proteinase, periodic acid, and neuraminidase. Sensitivities of antigens to trypsin, pronase, periodic acid, and neuraminidase were determined by the methods described elsewhere.[10] Colorectal cancer cell line SW1222[10] was used as a target cell for monoclonal antibodies 7R1-25-12-3, 7R1-110-9-7, and LISA 101. A small-cell lung carcinoma cell line SBC-5[10] was used as a target cell for monoclonal antibody 7R1-93-19-12. These cell lines were employed because they were found to express more of the antigens recognized by the corresponding monoclonal antibodies than the VMRC-LCR cell line which was originally used as an immunogen to produce the hybridomas.

抗原生化学検査

蛋白分解酵素，過ヨウ素酸及びノイラミニダーゼに対する感受性検査． 別報[10]に記述された方法によってトリプシン，プロナーゼ，過ヨウ素酸及びノイラミニダーゼに対する抗原の感受性を測定した．結腸直腸癌細胞株 SW1222[10] をモノクローナル抗体 7R1-25-12-3，7R1-110-9-7 及び LISA 101の標的細胞として用いた．肺小細胞癌細胞株 SBC-5[10] をモノクローナル抗体 7R1-93-19-12の標的細胞として用いた．これらの細胞株を使用したのは，ハイブリドーマを産生するのに免疫原として最初使用された VMRC-LCR 細胞株よりも対応するモノクローナル抗体により認識される抗原をより多く発現していたからである．

Determination of soluble antigen molecular weights. A pleural effusion derived from a case of pulmonary adenocarcinoma was fractionated upon gel filtration in TSK gel G 5,000 PWXL (Toyo Soda, Yamaguchi, Japan). The antigen content in the eluate fractions was measured by the sandwich-type ELISA technique described previously in this report.

可溶性抗原分子量の測定． 肺腺癌症例由来胸水を TSK gel G 5,000 PWXL（東洋曹達，山口）のゲル濾過上で分画した．本報で既述した sandwich 型 ELISA 法で溶出液分画の抗原含有量を測定した．

Staining of Tissue Sections. Formalin-fixed and paraffin-embedded tissue sections of a variety of cancer and normal tissues were stained with LISA 101 antibody using the indirect immunoperoxidase method (the avidin-biotin complex method), as previously described.[2]

組織標本の染色． 各種癌及び正常組織のホルマリン固定，パラフィン包埋の組織標本を，前報[2]で記述した間接免疫ペルオキシダーゼ法（アビジン-ビオチン複合法）を用いて LISA 101抗体で染色した．

RESULTS

Comparison of the Sensitivities between RI-ELISA and the Usual Sandwich-type ELISA. Shown in Figure 1 is the dilution curve of KL-6 antigen in RI-ELISA using unpurified KL-6 antibody in the KL-6 hybridoma culture supernatant as the immobilized antibody, as well as the dilution curve of a usual sandwich-type ELISA using directly purified KL-6 antibody. In both assays, specific reactions were observed at concentrations of 16 U/ml or more of KL-6 antigen. All the specific reactions at concentrations of 16, 32, and 64 U/ml were slightly higher in the RI-ELISA than in the usual sandwich-type ELISA.

結　果

RI-ELISA 法と通常の sandwich 型 ELISA 法の感度の比較．精製 KL-6 抗体を直接用いる通常の sandwich 型 ELISA 法の希釈曲線と，固相抗体として KL-6 ハイブリドーマ培養上清中の未精製 KL-6 抗体を用いる RI-ELISA 法による KL-6 抗原の希釈曲線とを図1に示した．両測定法において KL-6 抗原が16 U/ml 以上の濃度のとき特異性のある反応が観察された．KL-6 抗原濃度が16，32及び64 U/ml のとき，どの特異性反応も通常の sandwich 型 ELISA 法よりも RI-ELISA 法による方が若干高かった．

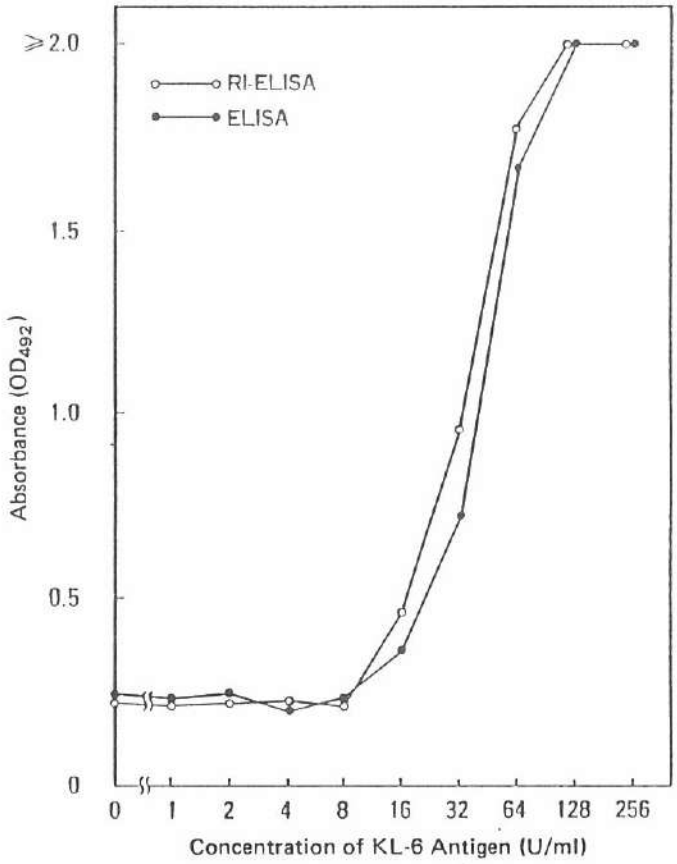

Figure 1. The sensitivity of RI-ELISA using the KL-6 antibody (○) was compared with that of the usual sandwich-type ELISA in which purified KL-6 antibody was directly used as the immobilized antibody (●). The nonspecific reactions observed at antigen concentrations of 8 U/ml or less were almost the same in both of the assays. However, the absorbances at antigen concentrations of 16, 32, and 64 U/ml were slightly higher in RI-ELISA than in the usual sandwich-type ELISA.

図1．KL-6 抗体（○）を用いる RI-ELISA 法の感度と，固相抗体（•）として精製 KL-6 抗体を直接使用する通常の sandwich 型 ELISA 法の感度を比較した．抗原濃度 8 U/ml 以下のとき認められた非特異性反応は，両測定法においてほぼ同一であった．しかし，抗原濃度が16，32及び64 U/ml のときの吸光度は通常の sandwich 型 ELISA 法よりも RI-ELISA 法の方がわずかに高かった．

Selection of Hybridomas by RI-ELISA Using the HRP-KL-6 Antibody. Figure 2 shows some of the results of selecting hybridomas by means of RI-ELISA. The KL-6 hybridoma culture supernatant was used as a positive control and that of the MOPC-21 myeloma cell was used as a negative control. The absorbance difference between the test culture supernatant and the negative control is ΔOD_{492}. The relative index is the ratio of ΔOD_{492}, developed against the adenocarcinoma antigens (from pleural effusions and sera), to that developed against control antigens (from tuberculosis inflammatory effusions and healthy sera), respectively.

In RI-ELISA of the KL-6 hybridoma culture supernatant, the relative index for the pleural effusion was more than 47.0 and that for the serum was more than 3.4. In the majority of the culture supernatants from 71 uncloned hybridomas, the relative indices were more than 1.2 for both the pleural effusion and serum as were indices of the 7R1-25, 7R1-93, and 7R1-110 hybridomas; but in only a few the relative index was about 1.0, similar to that of 7R1-103. Three uncloned hybridomas, whose relative indices were more than 1.5, were selected and cloned by the limiting dilution method followed by recloning. Eventually four hybridomas [7R1-25-12-3 (IgM), 7R1-93-19-12 (IgM), 7R1-110-9-7 (IgM), and 7R1-110-24-2 (LISA 101, IgG_1)] were established. All of them had relative indices of more than 2.5 for both pleural effusion and serum antigens. Since only the LISA 101 antibody belonged to IgG_1 and the others to IgM, the LISA 101 antibody was initially selected for further studies because purification of the IgG_1 antibody is usually easier than that of the IgM antibody. In addition, the conjugation of HRP is more effective in the IgG_1 antibody than in the IgM antibody.

Antigen Biochemical Properties. As shown in Table 1, the expression of the antigens on the cell surface of cell lines, as determined with the four mono-clonal antibodies was not changed by treatment of the cells with proteinases but was changed via degradation with periodic acid treatment. Both the antigens recognized with the 7R1-25-12-3 antibody and the LISA 101 antibody were sensitive to the treatment of neuraminidase, but those recognized with antibodies 7R1-93-19-12 and 7R1-110-9-7 were resistant. These results indicate that both 7R1-25-12-3 and LISA 101 antibodies

HRP-KL-6 抗体を用いた RI-ELISA 法によるハイブリドーマの選択. 図2は，RI-ELISA 法によるハイブリドーマの選択の結果を示している．陽性対照として KL-6 ハイブリドーマ培養の上清を，陰性対照として MOPC-21 骨髄腫細胞の上清を用いた．試験された培養上清と陰性対照の上清の吸光度差は ΔOD_{492} である．相対指数は，（胸水及び血清中の）腺癌抗原について算出された ΔOD_{492} の，（結核炎症性滲出液及び健康血清中の）対照抗原について算出された ΔOD_{492} に対する比である．

KL-6 ハイブリドーマ培養上清の RI-ELISA 法において，胸水の相対指数は47.0以上，血清の相対指数は3.4以上であった．71個の未クローン化ハイブリドーマの大部分の培養上清において，胸水及び血清の相対指数はハイブリドーマ7R1-25，7R1-93及び7R1-110の相対指数同様1.2以上であったが，ごく少数の培養上清において，相対指数は7R1-103の相対指数同様約1.0であった．相対指数が1.5以上である3個の未クローン化ハイブリドーマを選択し，限界希釈法を用いてクローン化した後に再クローン化した．最終的に4個のハイブリドーマ〔7R1-25-12-3（IgM），7R1-93-19-12（IgM），7R1-110-9-7（IgM）及び7R1-110-24-2（LISA 101，IgG_1）〕を確立した．それらのハイブリドーマの相対指数はすべて胸水抗原も血清抗原も2.5以上であった．LISA 101 抗体のみが IgG_1 に，他の抗体は IgM に属していたが，IgG_1 抗体の精製は通常 IgM 抗体の精製よりも容易なので最初に LISA 101 抗体を今後の研究のために選択した．更に，HRP の結合は IgM 抗体よりも IgG_1 抗体の方がより効率が良いためでもある．

抗原の生化学的特徴. 表1に示したように，四つのモノクローナル抗体で測定した細胞株の細胞表面上での抗原の発現は，細胞を蛋白分解酵素による処理では変化しなかったが，過ヨウ素酸処理による分解によって変化した．7R1-25-12-3 抗体及び LISA 101 抗体を用いて認識される抗原はいずれもノイラミニダーゼ処理に対して感受性を示したが，抗体 7R1-93-19-12 及び 7R1-110-9-7 で認識された抗原は耐性を示した．これらの結果は，7R1-25-12-3 及び LISA 101 抗体はいずれもシアル酸化糖鎖抗原決定

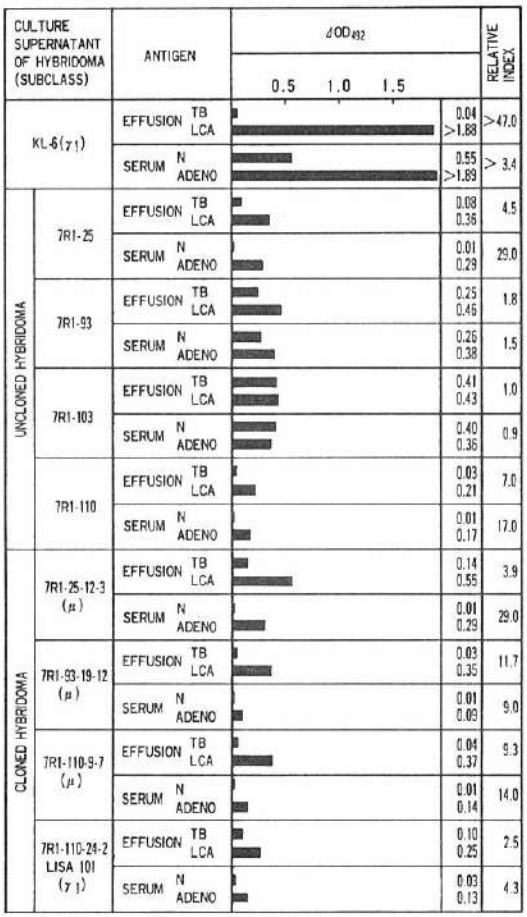

Figure 2. Selections of hybridomas were done by means of RI-ELISA. KL-6 hybridoma culture supernatant was used as a positive control and that of the MOPC-21 myeloma cells was used as a negative control. The absorbance difference between the test culture supernatant and the negative control is shown as ΔOD_{492}. The relative index is the ratio of ΔOD_{492}, developed against the adenocarcinoma antigens (from pleural effusions and sera) to that developed against control antigens (from tuberculosis inflammatory effusions and healthy sera), respectively. OD_{492} developed during RI-ERISA, using the HPR-KL-6 antibody, and the relative indices for pleural effusion antigens and serum antigens are shown. The highest values of OD_{492} were observed during RI-ELISA, when the KL-6 hybridoma culture supernatant and the antigen solutions of the adenocarcinoma effusion antigen or the adenocarcinoma serum antigen were examined. The hybridomas 7R1-25, R1-93, and 7R1-110 (all of which exhibited a relative index greater than 1.5) were cloned by means of the limiting dilution method. Afterwards, four cloned hybridomas, 7R1-25-12-3, 7R1-93-19-12, 7R1-110-9-7, and LISA 101 (7R1-100-24-2), were established, all of which exhibited relative indices greater than 2.5.

図2．RI-ELISA法によりハイブリドーマの選択を行った．陽性対照としてKL-6ハイブリドーマ培養上清を，陰性対照としてMOPC-21骨髄腫細胞の上清を用いた．試験された培養上清と陰性対照との吸光度の差異をΔOD_{492}として示した．相対指数は，（胸水及び血清の）腺癌抗原について算出されたΔOD_{492}の，（結核炎症性滲出液及び健康血清の）対照抗原について算出されたΔOD_{492}に対する比であり，HPR-KL-6抗体を用いたRI-ELISA法で算出されたOD_{492}及び胸水抗原相対指数と血清抗原相対指数を示している．RI-ELISA法を実施中KL-6ハイブリドーマ培養上清と腺癌胸水抗原又は腺癌血清抗原の抗原溶液を調べたときOD_{492}の最高値が認められた．ハイブリドーマ7R1-25，R1-93及び7R1-110（すべて1.5以上の相対指数を示した）を限界希釈法によりクローニングした．その後，四つのクローン化ハイブリドーマ7R1-25-12-3，7R1-93-19-12，7R1-110-9-7及びLISA 101（7R1-100-24-2）を確立したが，それはすべて2.5以上の相対指数を示した．

TABLE 1 ANTIGEN BIOCHEMICAL PROPERTIES

表1 抗体の生化学的特性

	Treatment with			
	Trypsin*	Pronase**	$NaIO_4$†	Neuraminidase‡
7R1-25-12-3	R	ND	S	S
7R1-93-19-12	R	ND	S	R
7R1-110-9-7	R	ND	S	R
LISA 101	R	R	S	S

R: resistant, ND: not determined, S: sensitive

Effects of enzymatic and chemical treatments on the bindings of monoclonal antibodies to target cells were examined. After living cells were treated with either 0.25% trypsin* or 0.25% pronase**, the cells were stained by means of the indirect fluorescent antibody method. After fixed cells were treated with several concentrations of either $NaIO_4$† or neuraminidase‡, the cells were stained by means of indirect enzyme immunoassay.

モノクローナル抗体の標的細胞との結合に及ぼす酵素及び化学処理の影響を調べた．生きた細胞を0.25%トリプシン* 又は0.25%プロナーゼ** で処理した後に，間接蛍光抗体法によって染色した．固定細胞を幾つかの濃度の $NaIO_4$† 又はノイラミニダーゼ‡ で処理した後に，間接酵素免疫検定法によって染色した．

react with sialylated carbohydrate antigenic determinant and the others with nonsialylated carbohydrate antigenic determinants.

Cross-Reactivity of Monoclonal Antibodies. Since two of the four monoclonal antibodies, LISA 101 and 7R1-25-12-3, appeared to react with sialylated carbohydrate antigens, their cross-reactivities were examined against antigenic determinants recognized by monoclonal antibodies, such as KL-6,[2] NS 19-9,[5] FH 6,[6] and LISA 101. While the LISA 101 antibody did not inhibit the binding of the NS 19-9 antibody, the 7R1-25-12-3 antibody inhibited NS 19-9 binding (Figure 3B). The 7R1-25-12-3 antibody, however, also inhibited binding of the LISA 101 antibody (Figure 3D). These antibodies failed to inhibit the binding of both the KL-6 antibody (Figure 3A) and the FH 6 antibody (Figure 3C). These results indicated that LISA 101, KL-6, and NS 19-9 antibodies reacted with different antigenic determinants, and the 7R1-25-12-3 antibody reacted with the determinants recognized by the LISA 101 and NS 19-9 antibodies.

Levels of LISA 1-6 in Sera. The levels of LISA 1-6 in sera are shown in Figure 4. The level was 8.5 ± 5.6 U/ml (mean ± SD) in 99 normal individuals. A cutoff level of 30 U/ml was established, at which only one of the normal individuals was considered to be positive at 1.0%

基と反応し，他の抗体は非シアル酸化糖鎖抗原決定基と反応することを示している．

モノクローナル抗体の交差反応性． 四つのモノクローナル抗体のうちの二つの抗体 LISA 101 及び 7R1-25-12-3 はシアル酸化糖鎖抗原と反応したように思えたので，KL-6,[2] NS 19-9,[5] FH 6[6] 及び LISA 101 などのモノクローナル抗体により認識された抗原決定基の交差反応性を調べた．LISA 101 抗体は NS 19-9 抗体の結合を阻止しなかったが，7R1-25-12-3 抗体は NS 19-9 結合を阻止した（図3 B）．しかし，7R1-25-12-3 抗体は LISA 101 抗体の結合も阻止した（図3 D）．これらの抗体は KL-6 抗体（図3 A）と FH 6 抗体（図3 C）の両抗体を阻止できなかった．これらの結果は，LISA 101，KL-6 及び NS 19-9 抗体は異なる抗原決定基と反応し，7R1-25-12-3 抗体は LISA 101 及び NS 19-9 抗体により認識された決定基と反応することを示した．

血清中の LISA 1-6 抗原値． 血清中の LISA 1-6 抗原値を図4 に示した．99名の健常者の抗原値は 8.5 ± 5.6 U/ml（平均 ± 標準誤差）であった．健常者のうち1名だけが1.0%（99名中1名）で陽性である30 U/ml を cutoff 値に設定した．肺扁平上皮

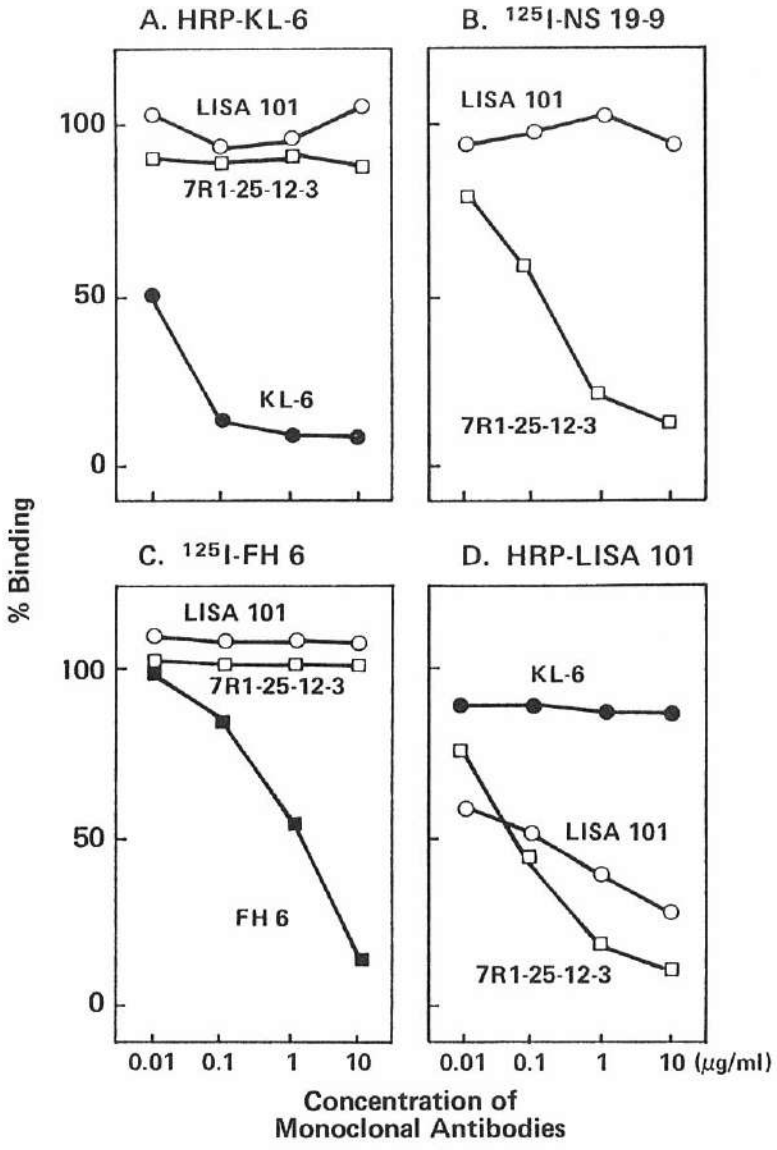

Figure 3. Cross-reactivities of monoclonal antibodies were examined by competitive inhibition assays. Both LISA 101 and 7R1-25-12-3 antibodies neither inhibited the binding action of the HRP-KL-6 antibody (A) nor the ^{125}I-FH 6 antibody (C). Though the 7R1-25-12-3 antibody competitively inhibited the binding of both the ^{125}I-NS 19-9 (B) and the HRP-LISA 101 (D) antibodies, the LISA 101 antibody failed to inhibit the binding of the ^{125}I-NS 19-9 antibody (B).

図3．競合阻止反応試験によってモノクローナル抗体の交差反応性を調べた．LISA 101 及び7R1-25-12-3 の両抗体ともに HRP-KL-6 抗体 (A) の結合作用も ^{125}I-FH 6抗体 (C) の結合作用も阻止しなかった．7R1-25-12-3 抗体は ^{125}I-NS 19-9 (B) と HRP-LISA 101(D) の両抗体の結合を競合的に阻止したが，LISA 101 抗体は ^{125}I-NS 19-9 抗体 (B) の結合を阻止しなかった．

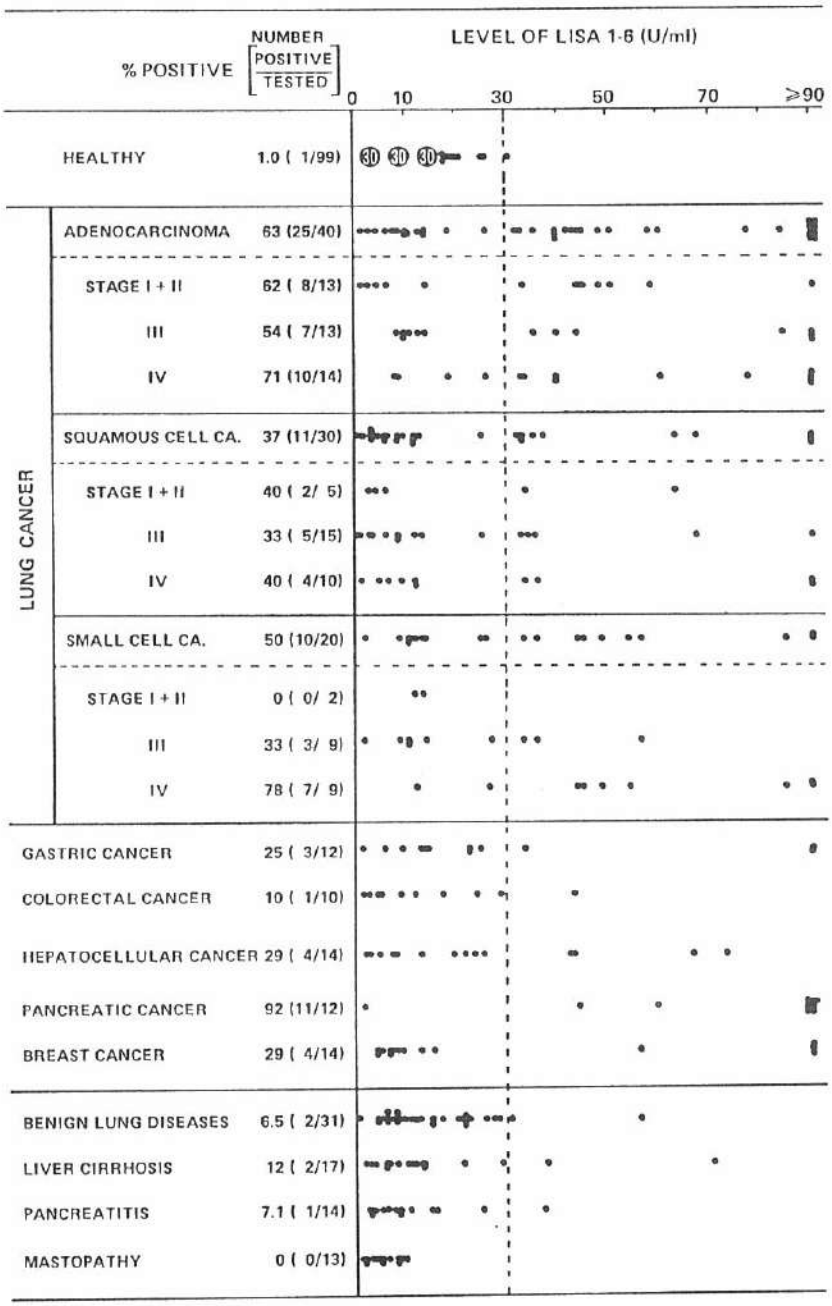

Figure 4. Levels of LISA 1-6 in the sera of healthy controls, cases of malignancies and benign diseases.

図4．健常対照者，悪性腫瘍症例及び良性疾患症例の血清中の LISA 1-6 抗原値.

(1/99). Patients with lung adenocarcinoma [63% (25/40)] were positive, as were 37% (11/30) of patients with lung squamous cell carcinoma, 50% (10/20) of patients with small-cell lung carcinoma, 25% (3/12) of patients with gastric cancer, 10% (1/10) of patients with colorectal cancer, 29% (4/14) of patients with hepatocellular cancer, 92% (11/12) of patients with pancreatic cancer, and 29% (4/14) of patients with breast cancer. At the clinical stage of lung adenocarcinoma, the percentage of those with elevated LISA 1-6 levels in sera was 62% (8/13) for clinical stages I and II, 54% (7/13) for clinical stage III, and 71% (10/14) for clinical stage IV.

In contrast, only 6.5% (2/31) of patients with benign lung diseases, including acute pneumonia, chronic bronchitis, and tuberculosis, were considered to be positive, and less than 15% of the patients with liver cirrhosis, pancreatitis, and mastopathy exhibited positive levels of LISA 1-6.

Levels of LISA 101 in Sera. As shown in Figure 5, serum levels of the LISA 101 antigen were examined in sera from healthy controls and from patients with malignancies whose levels of LISA 1-6 antigen had already been tested, as mentioned above. The level was 1.0 ± 0.95 U/ml in 44 normal individuals. Using a cutoff level 3.5 U/ml, one of the normal individuals was positive at 2.3% (1/44), as were 32% (10/31) of patients with lung adenocarcinoma, 27% (6/22) of patients with squamous-cell lung carcinoma, 22% (2/9) of patients with small-cell lung carcinoma, 40% (4/10) of patients with breast cancer, 36% (4/11) of patients with gastric cancer, 17% (2/12) of patients with colorectal cancer, 63% (5/8) of patients with pancreatic cancer, and 50% (5/10) of patients with hepatocellular cancer. Since the positive rate of the LISA 101 assay for lung cancer was much less than that of the LISA 1-6 assay, the clinical usefulness of the LISA 101 antigen might be less than that of the LISA 1-6 antigen.

Molecular Weights of Soluble Antigens. Upon gel filtration of a malignant pleural effusion, LISA 101 antigens appeared as three peaks in the 18th, 21st, and 24th fractions (Figure 6), while the KL-6 antigens appeared as two peaks in the 18th and 25th fractions. LISA 1-6 antigens also appeared as only two peaks in the 18th and 24th fractions. Mouse monoclonal IgM (900 K), ferritin (450 K), and BSA (67 K) were gel-filtrated as standard markers

癌患者では37％（30名中11名），肺小細胞癌患者では50％（20名中10名），胃癌患者では25％（12名中3名），結腸直腸癌患者では10％（10名中1名），肝細胞性癌患者では29％（14名中4名），膵臓癌患者では92％（12名中11名）及び乳癌患者の29％（14名中4名）と同様に，肺腺癌患者では63％（40名中25名）が陽性であった．肺腺癌の臨床病期別に血清中のLISA 1-6抗原値の上昇している患者の割合をみると，臨床病期ⅠⅠ及びⅡで62％（13名中8名），臨床病期Ⅲで54％（13名中7名），臨床病期Ⅳで71％（14名中10名）であった．

対照的に急性肺炎，慢性気管支炎及び結核を含む良性肺疾患患者のわずか6.5％（31名中2名）が陽性と考えられ，肝硬変患者，膵臓炎患者及び乳腺症患者ではLISA 1-6抗原値が陽性を示したのは15％以下であった．

血清中のLISA 101抗原値． 図5に示すように，健常者及び上述したようにLISA 1-6抗原値が既に測定された悪性腫瘍患者の血清中のLISA 101抗原値を調べた．44名の健常者においてその値は1.0±0.95 U/mlであった．Cutoff値3.5 U/mlを用いると，健常者のうちの1名が陽性で2.3％（44名中1名）の陽性率を示したが，肺腺癌患者で32％（31名中10名），肺扁平上皮癌患者で27％（22名中6名），肺小細胞癌患者で22％（9名中2名），乳癌患者で40％（10名中4名），胃癌患者で36％（11名中4名），結腸直腸癌患者で17％（12名中2名），膵臓癌患者で63％（8名中5名）及び肝細胞癌患者で50％（10名中5名）の陽性率を示した．LISA 101法による肺癌の陽性率はLISA 1-6法による陽性率よりもかなり低かったので，LISA 101抗原の臨床的有用性はLISA 1-6抗原よりも少ないであろう．

可溶性抗原の分子量． 悪性胸水のゲル濾過で，LISA 101抗原は第18分画，第21分画及び第24分画で各々ピークを示したが（図6），KL-6抗原は第18分画及び第25分画で各々ピークを示した．LISA 1-6抗原も第18分画及び第24分画で各々ピークを示した．マウスモノクローナルIgM（900 K），フェリチン（450 K）及びBSA（67 K）を分子量の標準マーカーとしてゲル濾過

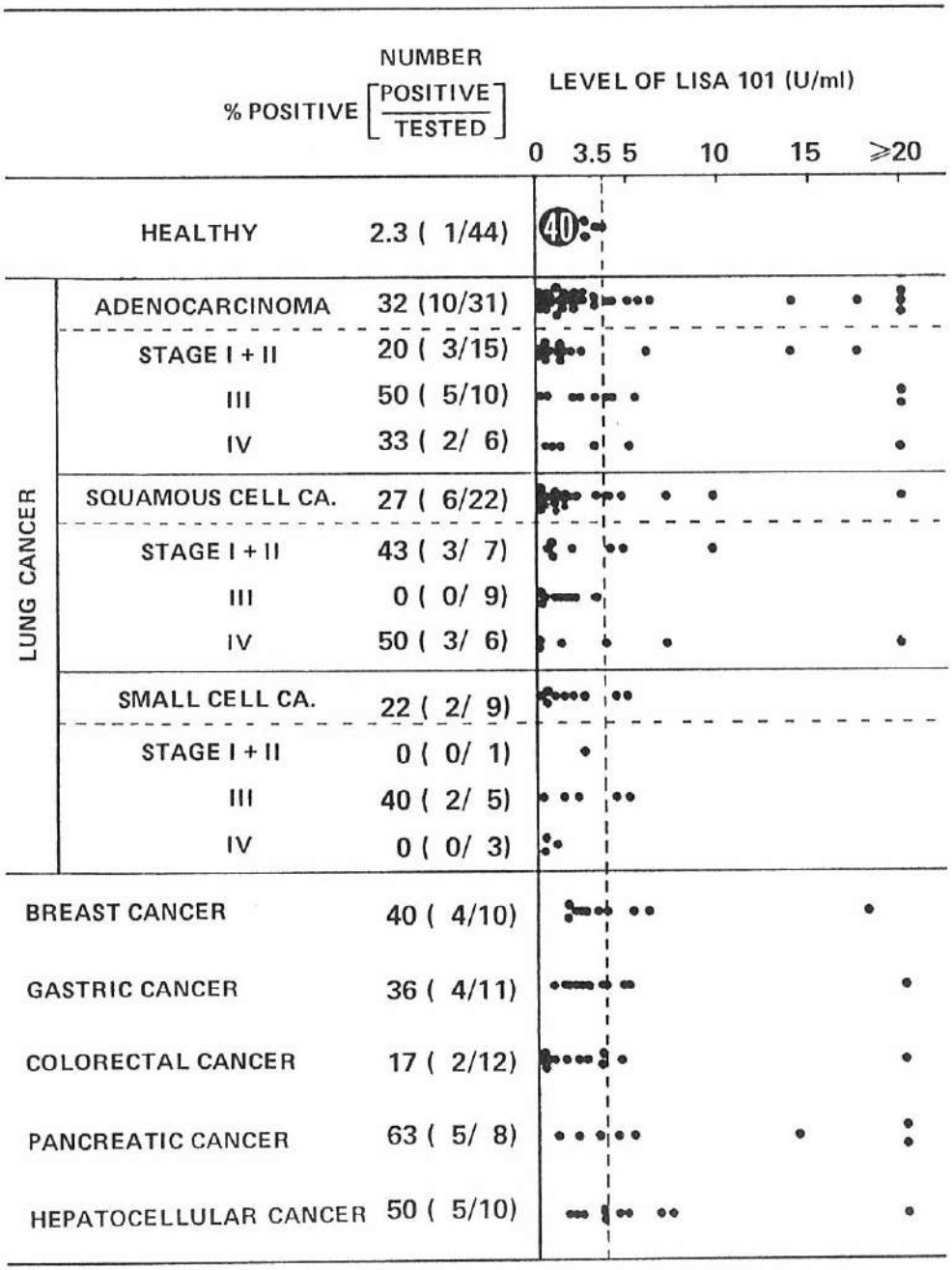

Figure 5. Levels of LISA 101 in the sera of healthy controls and cases of malignancies.

図5．健常対照者及び悪性腫瘍症例の血清中の LISA 101 抗原値.

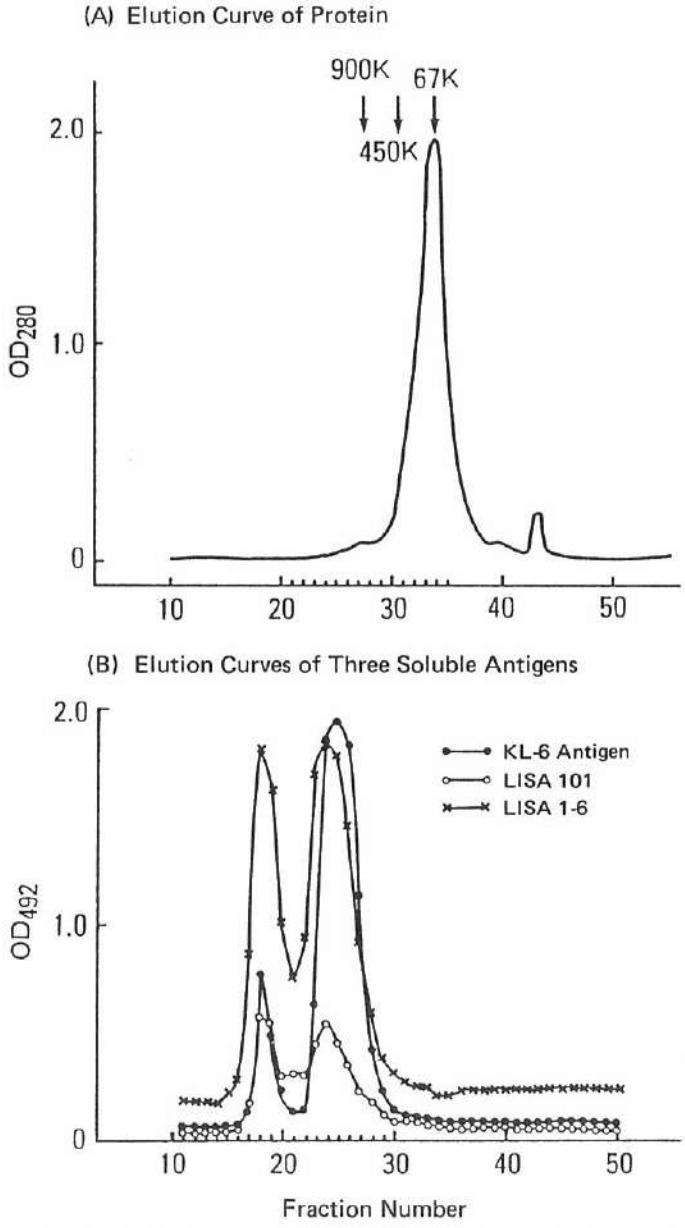

Figure 6. A pleural effusion derived from a pulmonary adenocarcinoma patient was gel-filtrated in TSK gel G 5,000 PWXL. A: The peaks of the protein elution curves of molecular weight standards, mouse IgM (900 K), ferritin (450 K), and BSA (67 K), were observed in the 27th, 30th, and 34th fractions, respectively. B: The levels of KL-6 antigen, LISA 101, and LISA 1-6 in each fraction of the eluted pleural effusion were measured by sandwich-type ELISA. KL-6 antigen exhibited two peaks in the void (18th) and 25th fractions. LISA 101 exhibited three peaks in the void, 21st, and 24th fractions. LISA 1-6 exhibited two peaks in the void and 24th fractions.

図6．肺腺癌患者由来胸水をTSK gel G 5,000 PWXLでゲル濾過した．A：分子量標準マーカーであるマウスIgM(900 K)，フェリチン(450 K)及びBSA(67 K)の蛋白質溶出曲線のピークが第27分画，第30分画及び第34分画でそれぞれ認められた．B：sandwich型ELISA法によって溶出胸水の各分画のKL-6，LISA 101及びLISA 1-6抗原値を測定した．KL-6抗原はvoid(第18)分画及び第25分画で二つのピークを示した．LISA 101抗原はvoid分画，第21分画及び第24分画で三つのピークを示した．LISA 1-6抗原はvoid分画及び第24分画で二つのピークを示した．

of molecular weight. These standard markers were eluted in the 27th, 30th, and 33rd fractions, respectively.

Reactivity of Monoclonal Antibodies with Tissue Sections. Reactivity with tissue sections of the monoclonal antibodies LISA 101, 7R1-25-12-3, and KL-6 is shown in Tables 2 and 3. The LISA 101 antibody stained five out of nine adenocarcinoma tissue sections (Figure 7A), five of seven squamous-cell carcinomas, and three of three small-cell lung carcinomas. The antigens were negative on tissue sections of type I and type II pneumocytes and bronchial epithelia, but they were positive on mucous cells (Figure 7D), and on mucus of the bronchial glands and apical border of the bronchiolar epithelia. It is interesting that the KL-6 antibody strongly stained all cancer tissue sections (Figure 7B) and serous cells and mucus of bronchial glands, but did not stain mucous cells of the bronchial glands (Figure 7F). The LISA 101 antibody strongly stained tissue sections of pancreatic cancer, breast cancer, gastric cancer, and colorectal cancer. It only slightly stained tissue sections of hepatocellular cancer.

した．これらの標準マーカーはそれぞれ第27分画，第30分画及び第33分画で溶出された．

組織標本に対するモノクローナル抗体の反応性． モノクローナル抗体 LISA 101，7R1-25-12-3 及び KL-6 の組織標本との反応性を表2及び3に示した．9例の腺癌組織標本のうち5例が LISA 101 抗体で染色され（図7A），7例の扁平上皮癌組織標本のうち5例が，3例の肺小細胞癌組織標本のうち全例が染色された．LISA 101 抗原はⅠ型及びⅡ型肺胞上皮と気管支上皮の組織標本では陰性であったが，気管支腺の粘液細胞（図7D）と粘液及び細気管支上皮の内腔側先端部では陽性であった．KL-6 抗体はすべての癌組織標本（図7B）と気管支腺の漿液細胞及び粘液を強く染色したが，気管支腺の粘液細胞（図7F）を染色しなかったことは興味深いことである．LISA 101 抗体は膵臓癌，乳癌，胃癌及び結腸直腸癌の組織標本を強く染色した．同抗体は肝細胞癌の組織標本を軽度にしか染色しなかった．

TABLE 2 REACTIVITY OF MONOCLONAL ANTIBODIES WITH MALIGNANT TISSUES
表2 モノクローナル抗体の悪性組織との反応性

Tissues		Monoclonal Antibodies		
		LISA 101	7R1-25-12-3	KL-6
Lung	Adenocarcinoma	+～++[a] (5/9)[b]	+～++ (4/9)	+～+++ (9/9)
	Squamous-cell carcinoma	+～++ (5/7)	+～++ (4/7)	+～+++ (7/7)
	Small-cell carcinoma	+～+++ (3/3)	+～++ (3/3)	++～+++ (3/3)
Pancreatic cancer		+～+++ (6/6)	+～+++ (5/6)	++～+++ (6/6)
Breast cancer		++～+++ (2/3)	+～++ (2/3)	+++ (3/3)
Gastric cancer		++ (2/2)	+～++ (2/2)	+～++ (2/2)
Colorectal cancer		+++ (2/3)	++～+++ (2/3)	+～++ (2/3)
Hepatocellular cancer		+ (1/1)	++ (1/1)	+ (1/1)

[a] Positive reactivity was scored based on the intensity of positively stained tissue sections as follows: +, weakly positive; ++, positive; +++, strongly positive.
以下のとおり陽性に染色された組織標本の強度に基づいて陽性反応性を分類した．+，弱陽性；++，陽性；+++，強陽性

[b] Positive number / tested number.
陽性数／測定数

TABLE 3 REACTIVITY OF MONOCLONAL ANTIBODIES WITH NORMAL TISSUES AND CELLS

表3 モノクローナル抗体の正常組織及び細胞との反応性

Tissues		Monoclonal Antibodies		
		LISA 101	7R1-25-12-3	KL-6
Lung	Type I pneumocyte	− (0/5)[a]	− (0/5)	− (0/5)
	Type II pneumocyte	− (0/5)	− (0/5)	++[b] (5/5)
	Bronchioles	+ (5/5)	+ (5/5)	++ (5/5)
	Bronchus	− (0/2)	− (0/2)	+ (2/2)
	Bronchial gland serous cell	− (0/2)	− (0/2)	+ (2/2)
	mucous cell	++ (2/2)	+ (2/2)	− (0/2)
Stomach	Surface mucous cell	++ (2/2)	++ (2/2)	− (0/2)
	Fundic gland	− (0/1)	− (0/1)	++ (1/1)
	Pyloric gland	− (0/1)	− (0/1)	− (1/1)
Colonic epithelium		+ (1/3)	+ (2/3)	− (0/3)
Pancreas	Acinus cell	− (0/2)	− (0/2)	− (0/2)
	Duct	++ (2/2)	++ (2/2)	++ (2/2)
	Islet cell	− (0/2)	− (0/2)	− (0/2)
Liver	Hepatocyte	− (0/2)	− (0/2)	− (0/2)
	Intrahepatic bile duct	++ (2/2)	++ (2/2)	+ (2/2)
Blood cells[c]	Granulocyte	− (0/2)	− (0/2)	− (0/2)
	Lymphocyte	− (0/5)	− (0/5)	− (0/5)
	Monocyte	− (0/2)	− (0/2)	− (0/2)

[a] Positive number / tested number. 陽性数/測定数

[b] Positive reactivity was scored based on the intensity of positively stained tissue sections as follows: +, weakly positive; ++, positive.
以下のとおり陽性に染色された組織標本の強度に基づいて陽性反応性を分類した．+，弱陽性；++，陽性

[c] Reactivity to blood cells were tested by means of indirect immunofluorescence staining.
血液細胞に対する反応性を間接免疫蛍光法により測定した．

Though the 7R1-25-12-3 antibody stained serial tissue sections similarly to the LISA 101 antibody, some discrepancies were noted. For some cancer tissues, the regions of the cells that were positively stained by the 7R1-25-12-3 antibody differed from those marked by the LISA 101 antibody. Differences in staining patterns were also observed in normal tissues, such as gastric surface mucous cells and colonic epithelium. Furthermore, the 7R1-25-12-3 antibody stained more weakly the mucous cells of the bronchial glands than did the LISA 101 antibody (Figure 7E). Peripheral blood mononuclear cells and granulocytes were not stained by any of these antibodies via the indirect immunofluorescent method.

LISA 101 抗体と同様に7 R1-25-12-3 抗体は連続組織標本を染色したが，幾つかの相違点が認められた．幾つかの癌組織については，7 R1-25-12-3 抗体により陽性に染色された細胞の部位は，LISA 101 抗体により染色された細胞の部位と異なっていた．胃粘膜上皮細胞や結腸上皮などの正常組織において，染色パターンの差も観察された．更に，7 R1-25-12-3 抗体は LISA 101 抗体の場合よりも気管支腺の粘液細胞の染色が軽度であった（図7 E）．間接蛍光抗体法による調査では，末梢血単核球及び顆粒球はこれらの抗体によって染色されなかった．

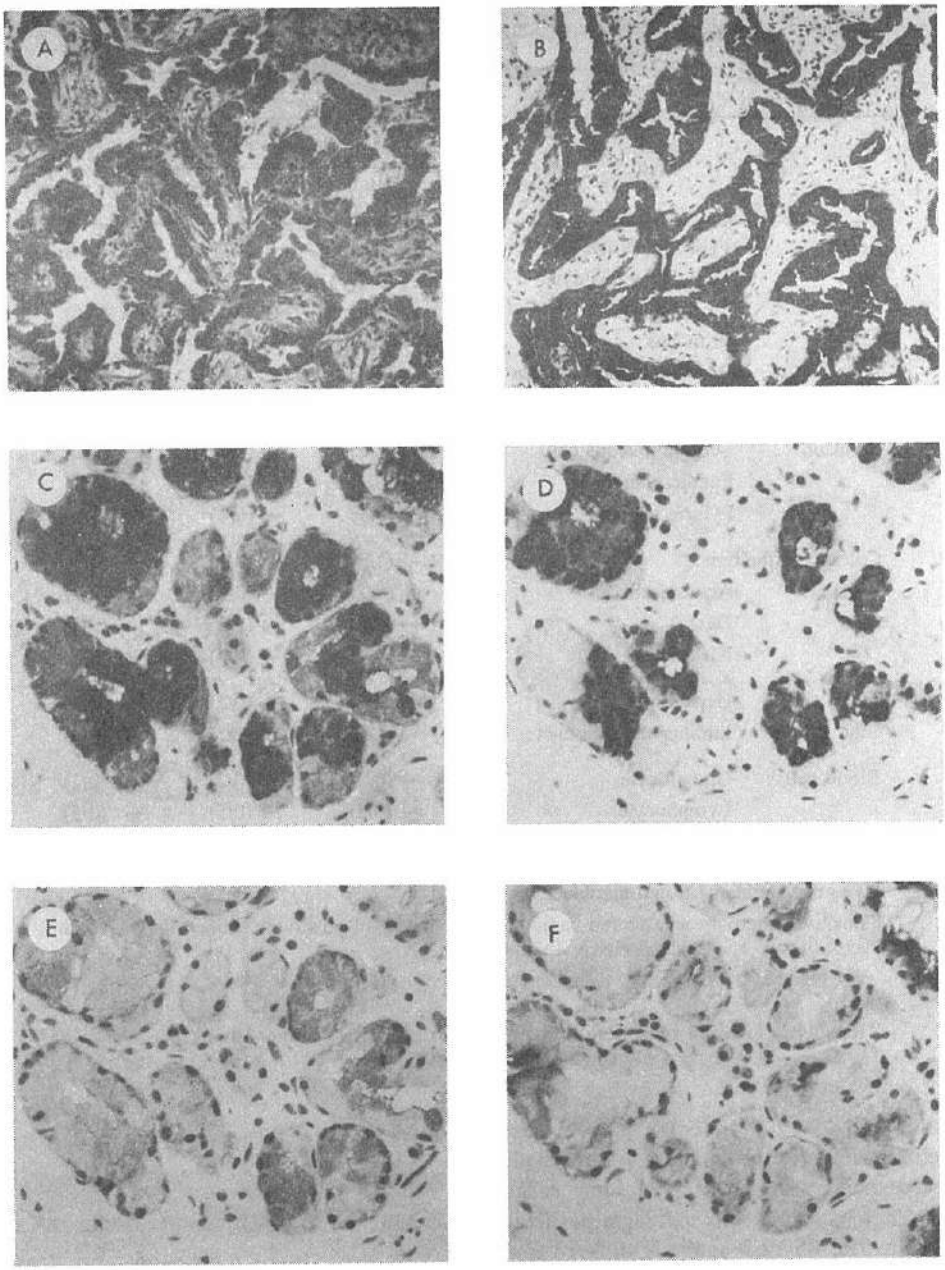

Figure 7. Immunoperoxidase staining (avidin-biotin complex method) of tissue sections with monoclonal antibodies. Both the LISA 101 (A) and KL-6 (B) antibodies stain the cell membrane and cytoplasm of lung adenocarcinoma. Both the LISA 101 antibody (D) and the 7R1-25-12-3 antibody (E) react with mucous cells, producing acidic mucin which is stained with alcian-blue on serial tissue sections (C). KL-6 antibody, on the other hand, reacts with serous cells of the bronchial gland, but not with mucous cells (F).

図7．モノクローナル抗体を用いた組織標本の免疫ペルオキシダーゼ染色（アビヂン-ビオチン複合法）．LISA 101(A)と KL-6 (B)の両抗体は肺腺癌の細胞膜及び細胞質を染色する．LISA 101 抗体 (D)も7R1-25-12-3 抗体 (E)も連続組織標本 (C)上でアルシアンブルーで染色された酸性ムチンを産出する粘液細胞に反応する．一方，KL-6 抗体は気管支腺の漿液細胞に反応するが，粘液細胞 (F) に反応しない．

DISCUSSION

A new screening method, RI-ELISA, was applied to select monoclonal antibodies capable of detecting circulating cancer-associated antigens. In RI-ELISA, since normal mouse serum contained in a buffered solution occupies the free reactive sites of the immobilized antimouse immunoglobulin antibody, the HRP-labeled antibody can effectively bind only to soluble antigen molecules, resulting in a high sensitivity. The sensitivity of RI-ELISA using the culture supernatant of the KL-6 hybridoma was nearly identical to that of the usual sandwich-type ELISA which uses purified KL-6 antibody as the immobilized antibody. Therefore, we assumed that RI-ELISA is a useful method for selecting new monoclonal antibodies reactive with soluble tumor antigens.

As a result of screening the hybridoma culture supernatants, four monoclonal antibodies were selected. Two of them, the 7R1-93-19-12 antibody (IgM) and the 7R1-110-9-7 antibody (IgM), appeared to react with nonsialylated carbohydrate antigens and the others, the 7R1-25-12-3 antibody (IgM) and the LISA 101 antibody (IgG_1), with sialylated carbohydrate antigens. From observations of cross-reactivities and tissue-staining patterns, both the 7R1-25-12-3 antibody and the LISA 101 antibody appeared to differ from previously reported monoclonal antibodies, such as KL-6,[2] FH 6,[6] C 50,[11] CSLEX-1,[12] and KM-93.[13]

However, the 7R1-25-12-3 (LISA 303) antibody inhibited the bindings of both the NS 19-9[4,5] and LISA 101 antibodies. The LISA 101 antibody, on the other hand, failed to inhibit the binding of the NS 19-9 antibody. These results indicate that the binding of the LISA 101 antibody and the NS 19-9 antibody takes place at different locations, regardless of whether the antigen determinants recognizing these antibodies exist on the same antigen. In contrast, the 7R1-25-12-3 antibody, cross-reacted with antigenic determinants which react with both the NS 19-9 and LISA 101 antibodies. Accordingly, one may consider two possible views of antigen recognition by these antibodies. Firstly, the antigenic determinants with which LISA 101, 7R1-25-12-3, and NS 19-9 antibodies react may exist on the same antigen, viz, the sialylated Le^a antigen. Or the LISA 101 antibody may react with sialylated carbohydrate antigens other than the sialylated Le^a antigens, whereas the 7R1-25-12-3 antibody may react with both sialylated carbohydrate antigens and

考　察

血中癌関連抗原の検出ができるモノクローナル抗体を選択するために，新しいスクリーニング法である RI-ELISA 法を適用した．RI-ELISA 法においては，緩衝液中に含まれる正常マウス血清が固相抗マウス免疫グロブリン抗体の自由反応部位を占めるので，HRP 標識抗体が効果的に結合できるのは可溶性抗原分子だけであり，その結果感受性が高くなる．KL-6 ハイブリドーマの培養上清を用いる RI-ELISA 法の感度は，固相抗体として精製 KL-6 抗体を用いる通常の sandwich 型 ELISA 法の感受性とほぼ同等であった．したがって，RI-ELISA 法は可溶性腫瘍抗原と反応する新しいモノクローナル抗体を選択する有用な方法であると考えた．

ハイブリドーマ培養上清のスクリーニングの結果，四つのモノクローナル抗体が選択された．そのうちの二つの 7R1-93-19-12抗体（IgM）と 7R1-110-9-7 抗体（IgM）は非シアル酸化糖鎖抗原に，残り二つの 7R1-25-12-3 抗体（IgM）と LISA 101 抗体（IgG_1）はシアル酸化糖鎖抗原に反応すると思われた．交差反応性及び組織染色パターンの観察から，7R1-25-12-3 抗体及び LISA 101 抗体の両抗体は KL-6，[2] FH 6，[6] C 50，[11] CSLEX-1 [12] 及び KM-93 [13] などの以前報告されたモノクローナル抗体とは異なると思われた．

しかし，7R1-25-12-3（LISA 303）抗体は，NS 19-9 [4,5] と LISA 101 の両抗体の結合を阻止した．一方，LISA 101 抗体は NS 19-9 抗体の結合を阻止しなかった．これらの結果は，LISA 101 抗体と NS 19-9 抗体の結合が，これらの抗体を認識する抗原決定基が同一抗原上にあるか否かにかかわらず，異なる場所で起こることを示している．対照的に，7R1-25-12-3 抗体は，NS 19-9 及び LISA 101 の両抗体と反応する抗原決定基と交差反応した．したがって，これら抗体の抗原認識について二つの見解が考えられるであろう．第一に，LISA 101，7R1-25-12-3 及び NS 19-9 抗体が反応する抗原決定基が同一抗原，すなわちシアル酸化 Le^a 抗原上に存在するかもしれない．あるいは LISA 101 抗体はシアル酸化 Le^a 抗原以外のシアル酸化糖鎖抗原と反応し，7R1-25-12-3 抗体はシアル酸化糖鎖抗原及びシアル酸化 Le^a 抗原と反応するかもしれない．これらの

sialylatead Le^a antigens. Despite these possibilities, LISA 101 was selected for further study since it has been suggested that the 7R1-25-12-3 antibody possesses reactivity quite similar to that of the NS 19-9 antibody, which detects CA 19-9 and is known as to be a useful tumor marker for pancreatic cancer. Although the 7R1-25-12-3 antibody inhibited the binding of the LISA 101 antibody, staining of a small number of serial sections suggested the following three possibilities for differences in reactivity: firstly, binding to different antigenic determinants; secondly, differences in binding affinity to the same antigenic determinant; and thirdly, the so-called masking phenomenon, during which the reaction of 7R1-25-12-3 antibodies — even though they are reactive to the same antigen — is inhibited by surrounding carbohydrate antigens due to binding at sites which are not exactly the same.

可能性にもかかわらず，CA 19-9 を検出し，膵臓癌の有用な腫瘍マーカーとして知られている NS 19-9 抗体の反応性と非常に類似した反応性を 7R1-25-12-3 抗体がもっていることが示唆されたので，今後の研究のために LISA 101 を選択した．7R1-25-12-3 抗体は LISA 101 抗体の結合を阻止したが，少数の連続標本の染色は，反応性の相違について三つの可能性があることを示唆した．第一に異なる抗原決定基との結合，第二に同一抗原決定基との結合能力の相違，第三にいわゆるマスキング現象である．すなわち，同一抗原分子上に反応するかどうかにかかわらず，7R1-25-12-3 抗体の結合は完全に同一ではない抗原決定基に反応するために周囲の糖鎖抗原によって阻害される可能性がある．

Bimonoclonal bideterminant assays, using the KL-6 antibody as the HRP-labeled antibody and the 7R1-93-19-12 or 7R1-110-9-7 antibodies as the immobilized antibodies were also assessed for their usefulness in clinical application (data not shown). Sera were derived from 30 healthy controls and 20 patients with lung adenocarcinoma whose LISA 1-6 antigen values were tested. Positive rates of lung adenocarcinoma were 20% (4/20) and 35% (7/20), respectively, when the cutoff values were set at the level of the mean ± 2 standard deviations of healthy controls. These data suggested that this assay was less useful when compared with LISA 1-6 antigen, so further study was not made.

HRP 標識抗体として KL-6 抗体を，固相抗体として 7R1-93-19-12 又は 7R1-110-9-7 抗体を用いる bimonoclonal bideterminant 法の臨床的応用の有用性についても評価した（データは示さない）．LISA 1-6 抗原値を測定した30名の健常対照者と20名の肺腺癌患者から血清を得た．健常対照者の平均±2 標準偏差の値で cutoff 値を設定すると，肺腺癌の陽性率は各々20%（20名中4名），35%（20名中7名）であった．これらのデータは，この測定法は LISA 1-6 抗原測定法ほど有用ではないことを示唆したので，以後この研究は行わなかった．

The LISA 101 antibody was able to detect at high rates circulating cancer-associated antigens, the molecular weight of which was more than 1,000 K. The positive rate of circulating antigens in patients with lung cancer was higher in a bimonoclonal bideterminant assay using the LISA 101 antibody together with the KL-6 antibody than in monoclonal bideterminant assays using the LISA 101 antibody or the KL-6 antibody alone.[2] Elevated serum levels of the LISA 1-6 antigen were observed in as many as 92% (11/12) of patients with pancreatic cancer, while the postive rate of CA 19-9 was only 75% (9/12) in the same patients. Moreover, only 7.1% (1/14) of patients with pancreatitis showed abnormal elevation of that antigen. Furthermore, the maximal value of pancreatitis was lower than the minimal level of the positive case with pancreatic cancer. The positive rate in 63% (25/40) of patients with

LISA 101 抗体は血中の癌関連抗原を高率で検出することができたが，その分子量は1,000 K 以上であった．肺癌患者の血中抗原の陽性率は，LISA 101 抗体又は KL-6 抗体のみを用いる monoclonal bideterminant 法よりも，KL-6 抗体と共に LISA 101 抗体を用いる bimonoclonal bideterminant 法の方が高かった．[2] 膵臓癌患者の92%（12名中11名）に血清中の LISA 1-6 抗原値の上昇が認められたが，同一患者の CA 19-9 陽性率はわずか75%（12名中9名）であった．更に，膵炎患者の7.1%（14名中1名）のみが LISA 1-6 抗原の異常な上昇を示した．なおかつ膵炎の最大値は膵臓癌陽性例の最小値よりも低かった．肺腺癌患者の63%（40名中25名）の陽性率は膵臓癌患者の陽性率より低かった．良性肺疾患患者の

lung adenocarcinoma was lower than that in patients with pancreatic cancer. This rate may be great enough to consider this antigen as a new marker for that disease, because a positive rate for patients with benign lung diseases was observed in only 6.5% (2/31) and abnormal levels were observed in 62% (8/13) of patients exhibiting clinical stages I and II of lung adenocarcinoma. Though these data are preliminary, the LISA 1-6 antigen may be promising for serological detection of adenocarcinomas of the lung and the pancreas.

The distribution patterns of antigens recognized by LISA 101 and KL-6 antibodies are noteworthy. The LISA 101 antibody stained only mucous cells but not serous cells from the normal bronchial gland which is the main organ secreting mucin in the lung. KL-6 antibody, on the other hand, stained only serous cells but not mucous cells. Despite this particular staining discrepancy, KL-6 antibody stained all cancer tissues that were positively stained by the LISA 101 antibody. These observations suggest that the two different carbohydrate antigens, most of which are originally produced by functionally different cells in the normal bronchial gland and form different mucins, are both produced in large quantities by transformed cells and may form an additional mucin molecule. The mucin molecule LISA 1-6 antigen may eventually increase in the sera of cancer patients. Furthermore, among various normal tissues, only the epithelia of bronchioles, pancreatic ducts, and bile ducts expressed both antigens simultaneously. These normal epithelia may produce and secrete a small quantity of LISA 1-6 antigen detected in the sera of healthy controls.

The usefulness of bimonoclonal bideterminant assay has already been demonstrated in a system for detecting a breast cancer-associated antigen, CA 15-3,[14] in which two monoclonal antibodies 115-D-8[15] and DF-3[16] are used against carbohydrate antigens. Our data also suggest that a bimonoclonal bideterminant assay using an appropriate pair of antibodies can increase both the specificity and sensitivity when compared with assays only using either of the other monoclonal antibodies. Since RI-ELISA is based on the principle of the bimonoclonal bideterminant assay, this method must be adequate for selecting new monoclonal antibodies as a suitable partner of already characterized monoclonal antibodies which detect circulating caner-associated carbohydrate antigens.

陽性率は6.5%（31名中2名）にのみ認められ，肺腺癌の臨床病期ⅠおよびⅡを示す患者の62%（13名中8名）に異常値が認められたので，この陽性率はこの抗原を腺癌の新しいマーカーとみなすのに十分と思われる．これらのデータは予備的なものであるが，LISA 1-6抗原は肺腺癌及び膵腺癌の血清学的検出に有望かもしれない．

LISA 101及びKL-6抗体により認識された抗原の分布パターンは注目すべきものである．LISA 101抗体は肺においてはムチンを産生する主要器官である正常気管支腺のうち粘液細胞のみを染色したが，漿液細胞は染色しなかった．一方，KL-6抗体は漿液細胞のみを染色し，粘液細胞を染色しなかった．この特殊な染色性の相違にもかかわらず，KL-6抗体は，LISA 101抗体により陽性に染色されたすべての癌組織を染色した．これらの観察結果は，その大部分が元来正常な気管支腺の機能的に異なる細胞により産出され，異なるムチンを形成すべき二つの異なる糖鎖抗原が両者ともに形質転換細胞により大量に産出され，更に新たなムチン分子を形成するかもしれないことを示唆している．そうしてムチン分子LISA 1-6抗原が，結局癌患者の血清中で増加するのかもしれない．更に，様々な正常組織の中で，細気管支，膵管及び胆管の上皮のみが同時に両抗原を発現した．これらの正常上皮は，健常対照者の血清中に検出されるLISA 1-6抗原を少量産出，分泌しているのかもしれない．

Bimonoclonal bideterminant法の有用性は乳癌関連抗原CA 15-3を検出する方法で既に証明されたが，[14] その方法では糖鎖抗原に対する二つのモノクローナル抗体115-D-8[15]とDF-3[16]を用いている．我々のデータは，一組の適切な抗体を用いるbimonoclonal bideterminant法がそれらの抗体のいずれか一つだけを用いる測定法と比べて特異性と感受性の両方を増大できることも示唆している．RI-ELISA法はbimonoclonal bideterminant法の原則に基づいているので，血中癌関連糖鎖抗原を検出する特異性既知のモノクローナル抗体の適切なパートナーとして新しいモノクローナル抗体を選択するにはこの方法が適切であるに相違ない．

It should be emphasized, furthermore, that RI-ELISA can be applied to the selection of monoclonal antibodies not only for use against cancer-associated carbohydrate antigens, but also for use against other soluble and solubilized antigens, such as other types of cancer-associated antigens, oncogene products, and plasma and membrane proteins. This is because a lot of numerous monoclonal antibodies, which exhibit high sensitivity, can be selected in a short time for use against additional antigenic determinants on a soluble molecule recognized by an already characterized monoclonal antibody.

更に，癌関連糖鎖抗原に対するモノクローナル抗体の選択に使用するばかりでなく，他の種類の癌関連抗原，癌遺伝子及び血漿並びに膜蛋白質などの他の可溶性及び可溶化抗原に対するモノクローナル抗体の選択にもRI-ELISA法が応用できることを強調すべきである．なぜならば，特異性既知のモノクローナル抗体により認識される可溶性分子上の別の抗原決定基に対する高い感受性を示す多数のモノクローナル抗体を短期間に選択することができるためである．

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