A Human Tumor-Specific Antigen from Lung Cancer
——A New Purification Method——

Masayoshi KUWABARA, Yoshito MATSUBARA* and Sadao IKEDA*

The Department of Chest Diseases and Thoracic Surgery
Kansai-Denryoku Hospital, Osaka 553, *JAPAN*
**Chest Diseases Center, Kyoto-Katsura Hospital, Kyoto* 615, *JAPAN*
Masayoshi KUWABARA Kansai-Denryoku Hospital
2-1-7, *Fukushima, Fukushimaku, Osaka,* 553 *JAPAN*

(Received, April 15, 1986)

SUMMARY

A new purification method for tumor-specific antigens is described. The TS-1 antigen was purified with ammonium sulfate and fractionation by column chromatography, Sephadex G 50, G 200 and Con A Sepharose 4B. It was shown using the double immunodiffusion test that the TS-1 and CEA had a common antigenic determinant.

The TS-2 antigen, which was purified by a simpler method using proteolysis of Pronase E and fractionation by column chromatography, was also a tumor-specific antigen. Anti-TS-1 serum had crossreactivity with TS-2 antigen, but anti-CEA and anti-NCA had no crossreactivity in double gel diffusion with TS-2 antigen.

It was shown using the immunofluorescence test and enzyme-labelled antibody technique that TS-1 and TS-2 antigens localized only on the cancer cell surface. They were not localized on the normal tissue of bronchial epithelium, mucous glands, vascular tissue or alveoli. The sedimentation coefficients of TS-1 and TS-2 antigens, which were measured by boundary ultracentrifugation, were 6.81 and 1.65, respectively. Sugar parts of the TS-1 antigen were composed of fucose, galactose, glucose, N-acetylglucosamine and sialic acid, while those of the TS-2 antigen were composed of fucose, galactose, N-acetylgalactosamine and unknown sugars. The protein parts of the TS-1 and TS-2 antigens accounted for 11.3% and 15.1%, respectively. While TS-1 antigen and CEA showed affinity to Con A, TS-2 antigen did not. These results suggested that TS-1 and TS-2 antigens were cancer specific and that TS-1 antigen was divided by the proteolysis with Pronase E into TS-2 antigen and NCA.

INTRODUCTION

Recent years have seen an increasing number of reports on the presence of tumor-associated antigens in human lung cancers and methods for their purification[1-6]. The TS antigen, which was found by Ikeda et al, is one such tumor-associated antigen[7-8]. It is a mucoprotein and is extracted from lung cancer specimens using the method of Winzler for isolation of mucoprotein from serum with ammonium sulfate[9], and is purified by column chromatography. It was also

confirmed using the immunofluorescence test and enzyme-labelled antibody technique that TS antigen is localized on tumor cell surface[10]. It was also verified that the TS antigen has a similar antigenic determinant to CEA.

Here, we report details of the purification methods and the characteristics of TS antigens : TS-1 antigen, which is a more highly purified TS antigen, and TS-2 antigen, which is purified by a simpler, easier way.

MATERIALS AND METHODS

I. Purification of Tumor-specific-1 antigen (TS-1 antigen)

Preparation of crude TS-1 antigen by ammonium sulfate

Solid tumors, which contained different histological types of lung cancer, were obtained at surgery and immediately frozen at −20°C until use. These tumor materials were thawed and homogenized with 2 volume of distilled water. The homogenate was centrifuged for 30 minutes at 4°C and 10000 Xg. The pellet was then discarded. The supernatant was collected as tumor extract.

Further preparation of the antigen was performed using the method of Winzler for isolation of mucoprotein from serum with ammonium sulfate[9].

The supernatant, to which was added one-tenth volume of 1 M sodium acetate, was adjusted with ammonium sulfate to final concentration of 2. 73 M. The mixture was again centrifuged, and the pellet was discarded. The pH of the supernatant was brought to 4. 9 with 1 N hydrochloride solution. The mixture was again centrifuged and the supernatant was collected. The pH of the supernatant was further acidified to 3. 6 with a 1 N hydrochloride solution. The mixture was centrifuged. The supernatant was satured with ammonium sulfate and hled at 4°C for 48 hours. Then, the supernatant was centrifuged and the pellet was dialyzed and lyophilized. This was named crude TS-1 antigen (TS-1 antigen).

Purication of TS-1 antigen

The crude TS-1 antigen was purified by fractionation with column chromatography. At first, the solution of crude TS-1 antigen was separated on a column of CM Sephadex (G50) and the column was washed out with 0. 1 M acetate buffer (pH 4. 0) containing 0. 5 M Nacl. The eluted first fraction was dialyzed and lyophilized. Then, this fraction was again separated on Sephadex G-200 and the first fraction, which was eluted in saline, was dialyzed and lyophilized. This first fraction was applied to a column of Con A Sepharose 4B and the column was washed out with 0. 02 M phosphated buffer (pH 7. 0) containing 1M Nacl. The column was incubated for 12 hours with 1 M alpha-methyl-D-glucoside in phosphate buffer and washed out with the same buffer solution. Finally, this Con A linked fraction was separated on a column of Sepharose 4B. The eluted first fraction is the purified TS-1 antigen (Figure 1).

Production of antiserum

Antiserum to TS-1 antigen was produced in rabbit. One mg of TS-1 antigen, which was emulsified in complete Freund adjuvant, was injected subcutaneously into the back of rabbit. Six weeks later, the rabbit serum was obtained and the gamma-globulin fraction was prepared with semi saturated ammonium sulfate. This 5% saline solution of anti-TS serum was absorbed

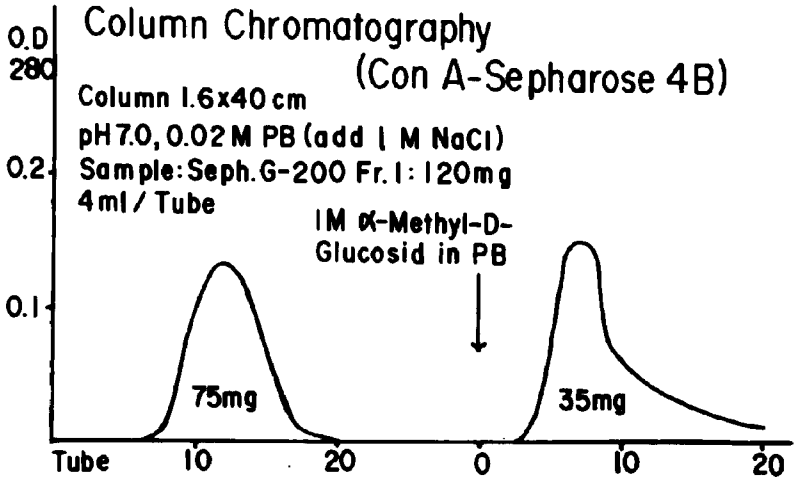

Fig. 1 Con A-Sepharose 4B chromatography of TS-1 antigen preparation

with a 10% saline solution of lyophilized normal lung extract with an incubation time of 1 hour at 37°C and then, for 12 hours at 4°C. The anti-TS-1 serum was also absorbed with pooled human serum of A, B groups under identical conditions. The mixture was centrifuged and the supernatant was used as the anti-TS-1 serum.

II. Purification of Tumor-specific-2 antigen (TS-2 antigen)

Purification of TS-2 antigen by proteolysis with Pronase E

The tumor materials, which were collected at the same time as those which underwent purification for TS-1 antigen, were thawed and homogenized with 2 volumes of distilled water. Then, the tumor homogenate was mixed with 1 N solution of sodium hydroxide to a final concentration of 0. 1 normal and dissolved with 1% sodium azide to a final concentration of 0. 2%. The homogenate was incubated for 48 hours at 4°C. The pH of this homogenate was adjusted to 7. 5 with 1 N solution of hydrochloride and 0. 1 M tris-hydrochloride buffer (pH 8. 0). Then, the homogenate was exposed to the Pronase E at the rate of 3 mg per gram of tumor wet weight.

This homogenate was incubated for 12 hours at 37°C and dissolved. After incubation, the pH of the homogenate was brought to 7. 5 with the 0. 1 M tris-hydrochloride buffer (pH 8. 0) and Pronase E was again added as before. Incubation was performed for 12 hours at 37°C. The homogenate was digested and filtrated. The filtrate was saturated with ammonium sulfate and centrifuged. The pellet was dialyzed. After adding ethanol in a final concentration of 50%, it was again centrifuged. The pellet was dialyzed and lyophilized. The 5% solution of this extract was applied to a column of Sephadex G-50 and washed with saline. The first fraction was then separated on a column of Sephadex G-200 and the first fraction considered TS-2 antigen.

The TS-2 antigen was also purified from gastric cancer using the same method.

Production of antiserum

The antiserum to TS-2 antigen was produced by the same method as for the anti-TS-1 serum. Absorption with normal lung extracts and pooled human serum of A, B groups were also performed.

III. Specificity and antigenicity of the TS-1 and TS-2 antigens

Anti-TS-1 serum was tested for titer and specificity against TS-1 antigen and extracts of normal lung, liver, kidney and fetal liver tissues by the gel diffusion precipitation reaction.

Anti-TS-2 serum was also tested for titer and specificity against anti-CEA and anti-NCA by the same method. The TS-2 antigen from gastric cancer was also tested for antigenicity and specificity.

IV. Localization of TS-1 and TS-2 antigens

The localization of TS-1 and TS-2 antigens were histologically determined by the immunofluorescence test and enzyme-labelled antibody technique. The details of the method used have been described by Matsubara[10].

V. Analysis of the TS-1 and TS-2 antigens

The TS-1 and TS-2 antigens were treated with 2-mercaptoethanol and electrophoresis was performed in acrylamide gels with SDS in phosphate buffer. The sedimentation value of the antigens were also measured by the Beckmann Spinco Model.

The carbohydrate and amino acids composition of the TS-1 and TS-2 antigens were analyzed.

RESULTS

There was no reactivity between anti-TS-1 serum and extracts of normal lung, liver, kidney or fetal liver. Anti-TS-1, anti-CEA and anti-NCA sera showed precipitation line patterns identical with TS-1 antigen, indicating the existence of a common antigenic determinant among them. The precipitation line between anti-TS-1 serum and TS-1 antigen was distinct (Figure 2). Figure 3 shows the precipitation reactions of TS-2 antigen purified from lung cancer and gastric cancer

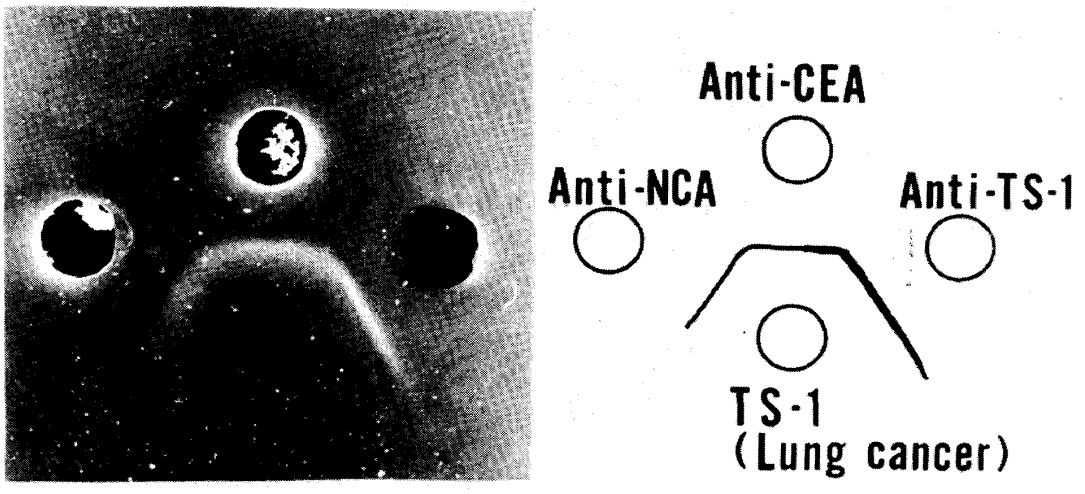

Fig. 2 Double immunodiffusion test of TS-1 antigen against anti-NCA, anti-CEA and anti-TS-1 serum.

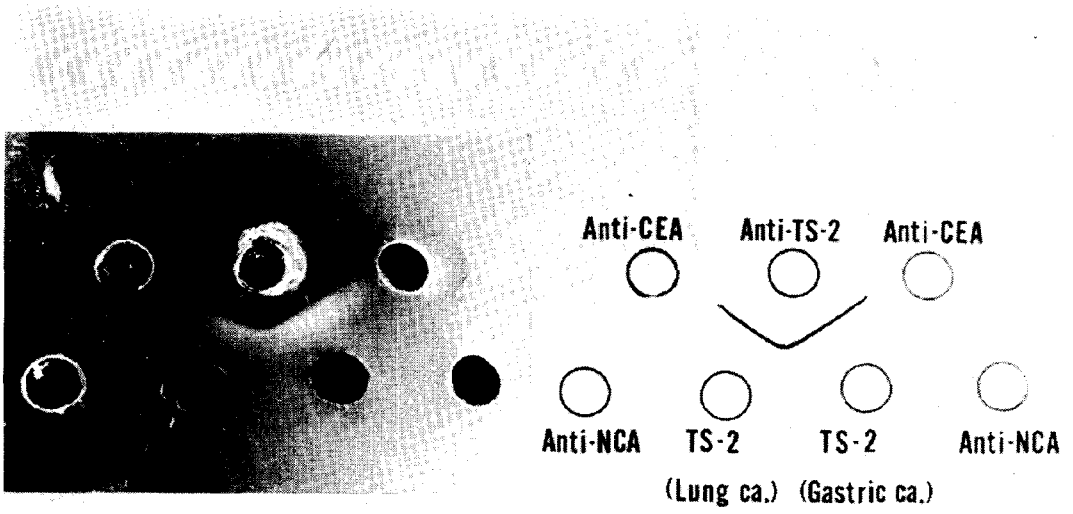

Fig. 3 Double immunodiffusion test of TS-2 antigen against anti-NCA, anti-CEA and anti-TS-2 serum.

against anti-CEA and anti-NCA sera. Neither TS-2 antigen from lung cancer nor TS-2 antigen from gastric cancer reacted with anti-CEA sera. A precipitation line was observed only between anti-TS-2 serum and both TS-2 antigens. The precipitation lines of both TS-2 antigens fused (Figure 3).

Specific fluorescence by the indirect immunofluorescent staining of lung cancer with anti-TS-1 serum was observed on cancer cell surfaces of all histological types of lung cancer. It was especially prominent in adenocarcinoma (Figure 4). However, there was no specific fluorescence in normal tissues of bronchial epithelium, mucous glands, vascular tissue or alveoli. By immunoperoxidase staining, only the cancer cell surfaces were stained (Figure 5). Further, by immunoelectron microscopy and staining with peroxidase-labelled anti-TS-1 serum, it was shown that the antigen-antibody complex was localized on the cancer cell surfaces (figure 6).

The specific fluorescence of lung cancer with anti-TS-2 serum as well as that of anti-TS-1 serum was observed on cancer cell surfaces of different histological types of lung cancer (Figures 4, 5, 6). There was no specific fluorescence with anti-TS-2 serum in normal tissues of bronchial epithelium, mucous glands, vascular tissue or alveoli. In gastric cancer, specific

Table 1. Carbohydrate composition

Sugar	TS-1 antigen	TS-2 antigen
Fucose	17.90%	6.38%
Mannose	Trace	
Galactose	31.32%	43.06%
Glucose	7.40%	
N-acetylglucosamine	37.03%	33.63%
Sialic acid	6.35%	
Unknown sugars		16.39

Analyzed by Prof. Yamakawa, Tokyo Univ.

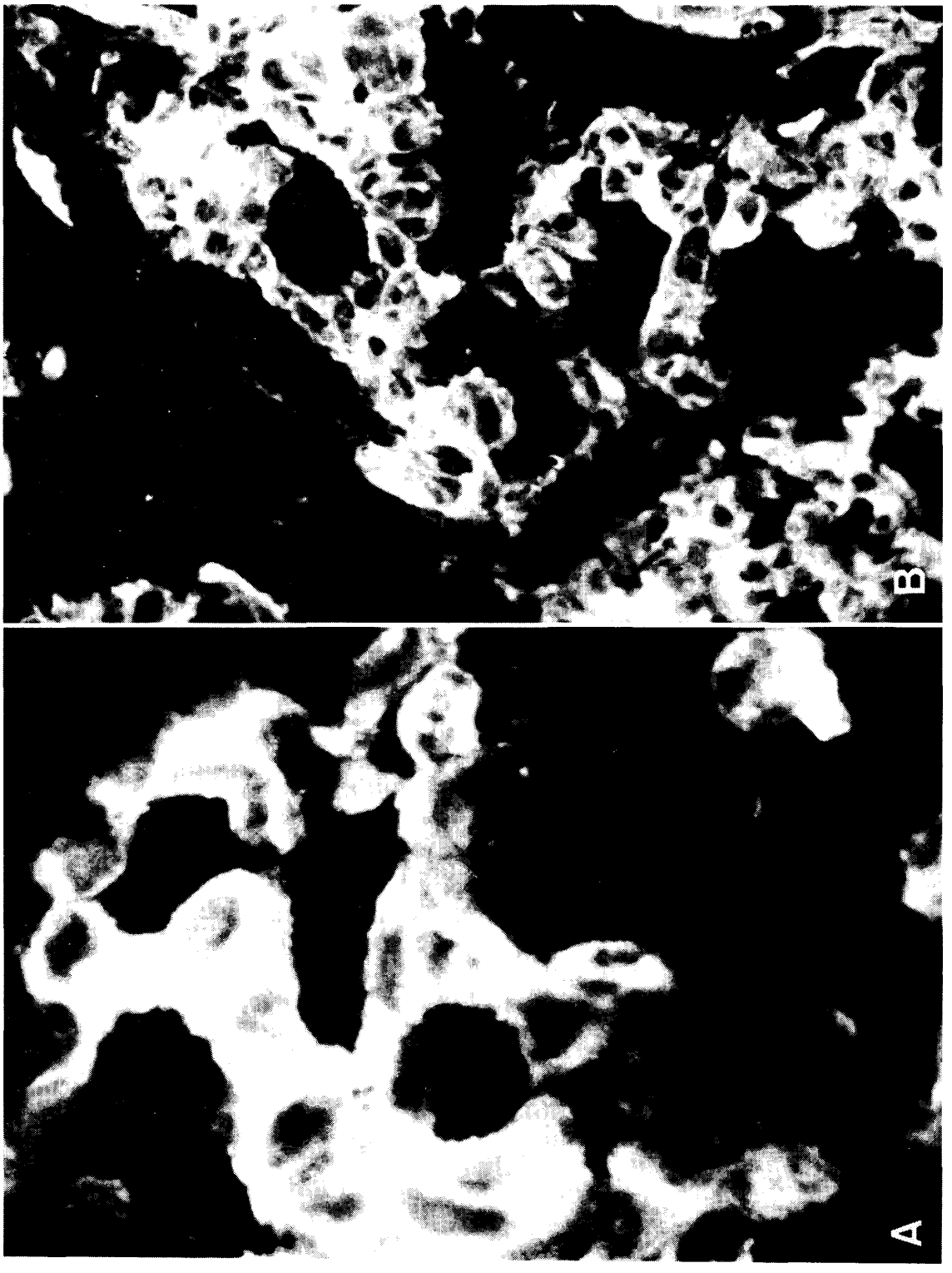

Fig. 4 Indict immunofluorescent staining of adenocarcinoma.
A: Anti-TS-1 serum (×400)
B: Anti-TS-2 serum (X200)

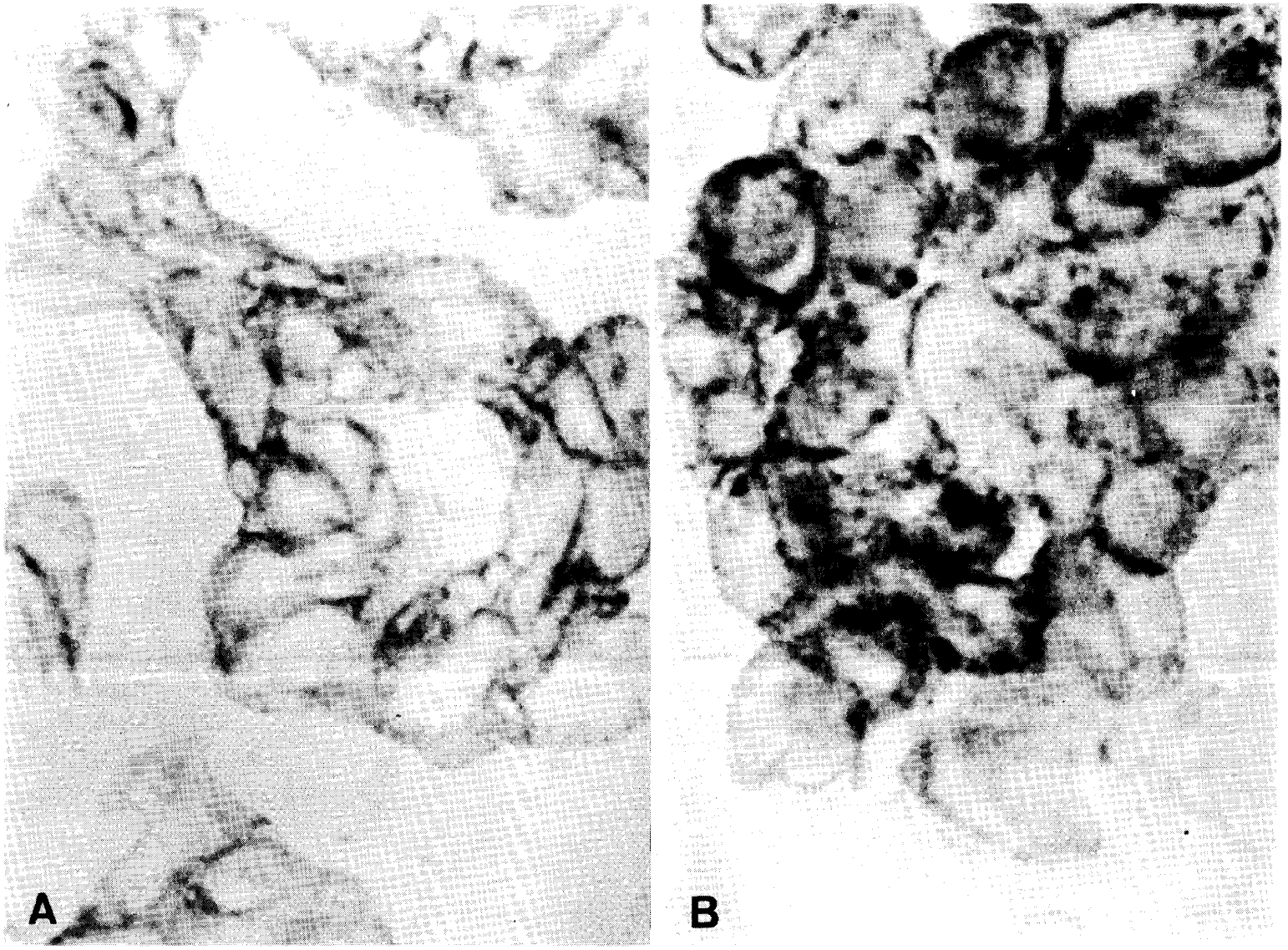

Fig. 5 Immunoperoxidase staining of adenocarcinoma of the lung.
A: Anti-TS-1 serum (×400)
B: Anti-TS-2 serum (×400)

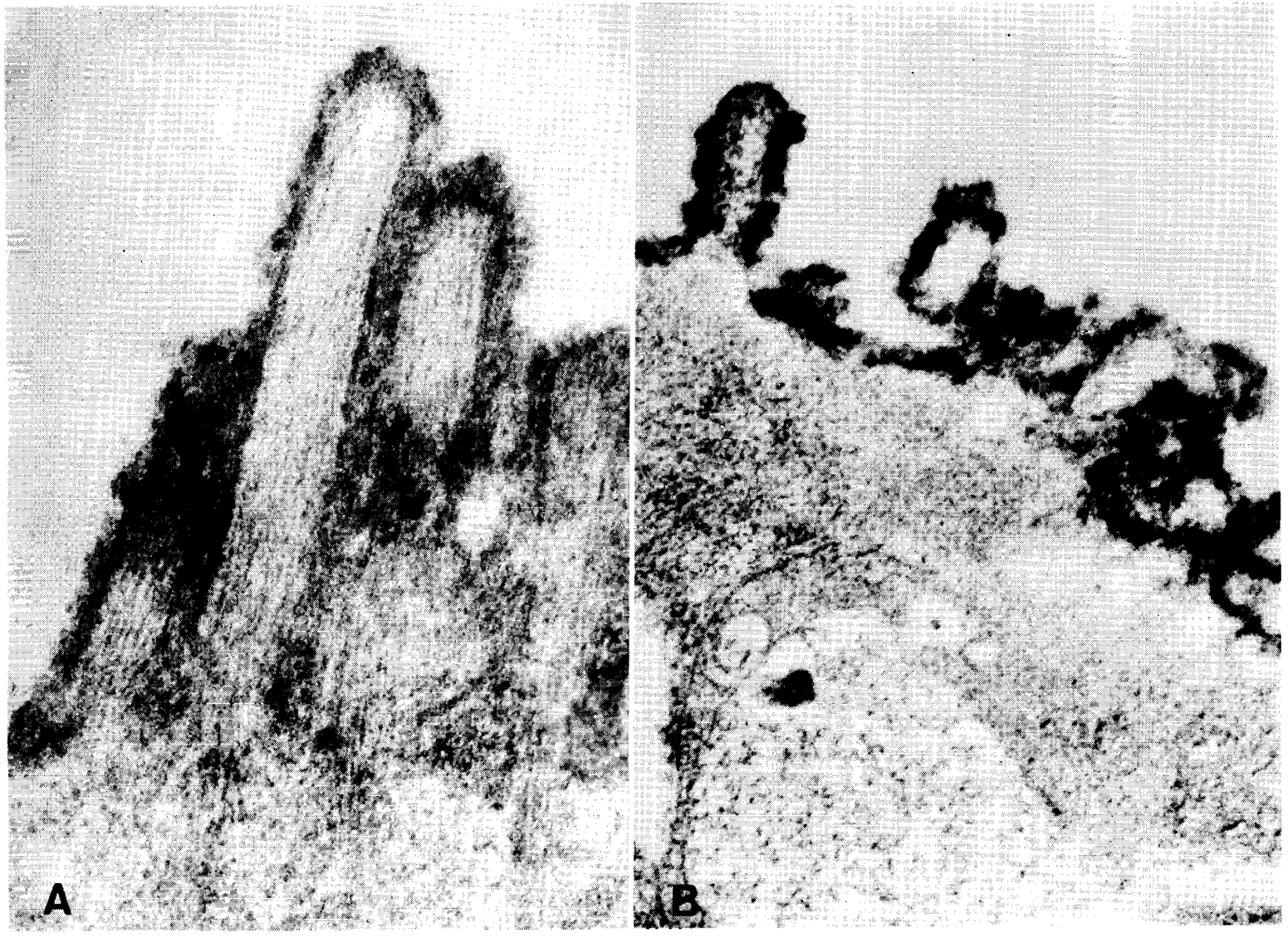

Fig. 6 Immuno-electron microscopy staining with peroxidase-labelled anti-TS-1 serum (A) and anti-TS-2 serum (B). The antigen-antibody complexes are localized only on the cancer cell surfaces. A: ×60000 B: ×36000

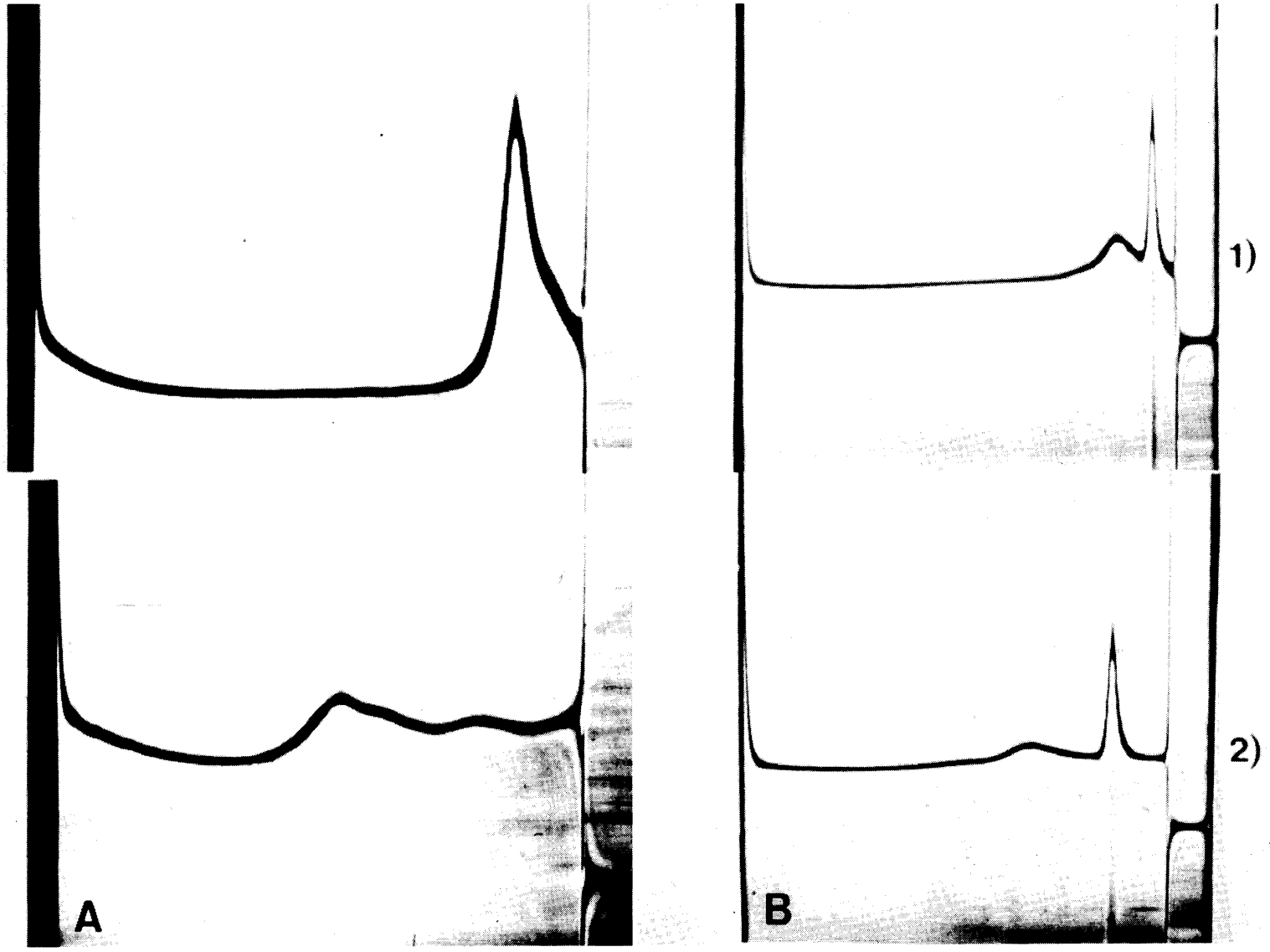

Fig. 7 Ultracentrifugal analysis of TS-1 and TS-2 antigens. A single peak is levealed in both cases. A: TS-1 antigen B: TS-2 antigen

Table 2. Amino acid composition of TS-1 and TS-2 antigen

Amino acid	% TS-1 antigen	%TS-2 antigen
Lysine	0.31	0.78
Histidine	0.59	0.30
Arginine	0.35	0.31
Asparatic acid	0.67	1.67
Threonine	2.01	1.48
Serine	1.35	1.26
Glutamic acid	0.81	0.91
Proline	1.05	0.55
Glycine	0.38	1.77
Alanine	0.54	0.78
Cysteine	0.22	(1/2 Cyst.)
Valine	0.94	0.65
Methionine	0.59	0.01
Isoleucine	0.54	1.93
Leucine	0.54	1.16
Tyrosine	0.14	0.95
Phenylalanine	0.32	0.58
Total	11.35	15.09

% TS (TS-1, TS-2), percentage of individual amino acid as a portion of the total weight of TS analyzed.

fluorescence was also observed on cancer cell surfaces.

The electrophoretic mobilities of TS-1 and TS-2 antigen on SDS acrylamide gel migrated near to the gamma-globulin region.

By ultracentrifugal analysis, TS-1 and TS-2 antigens each revealed the presence of a single peak. The sedimentation constants of TS-1 and TS-2 antigens were 6.81 S and 1.65 S, respectively (Figure 7).

Carbohydrate and amino acid compositions are summarized in Tables 1 and 2.

DISCUSSION

The TS antigen, which was purified by Ikeda et al[7-8], is a tumor-specific antigen found on tumor cell surfaces of lung cancer[10]. It was confirmed that the TS antigen had an antigenic determinant in common with CEA by the gel diffusion precipitation reaction.

In our studies of TS antigen, in order to determine its antigenic properties, we treated the TS antigen with periodate to degrade the sugars in glycoproteins and with Pronase E, a proteolytic enzyme, for the proteolysis of glycoproteins. In gel diffusion, the antigenicity of TS antigen against anti-TS serum was lost by the degradation of sugars, but was not lost by the proteolysis of proteins (Figure 8). This finding can be explained by assuming that the antigenic determination of the TS antigen was based on the sugars in glycoproteins. Thus, the TS antigen that was purified with ammonium sulfate and fractionated by column chromatography was named TS-1 antigen. Proteolysis of Pronase E was applied to the purification of tumor specific-antigen and we developed a simpler method for the purification of TS antigen. This was

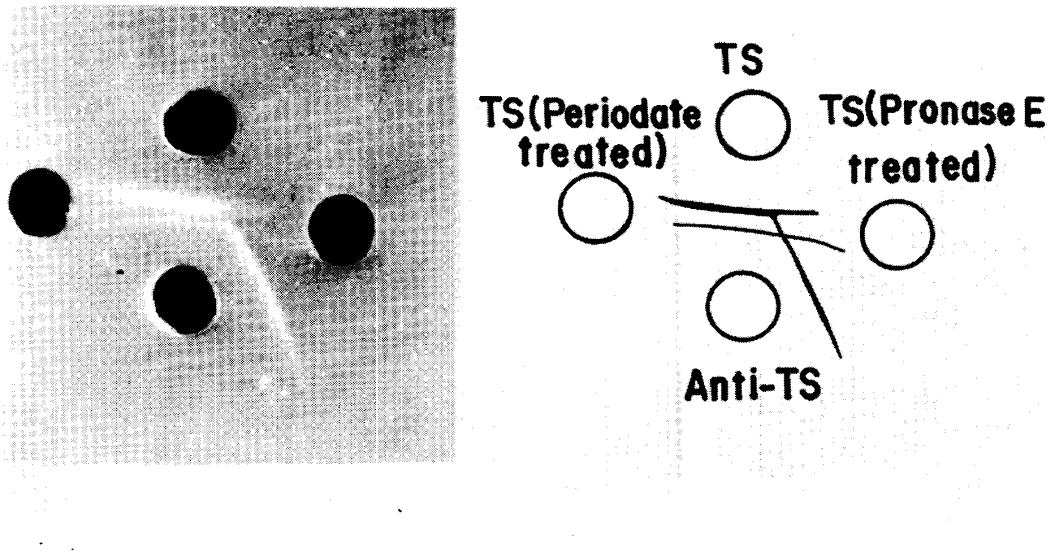

Fig. 8 Double immunodiffusion test of TS-antigens treated with periodate and Pronase E.

dubbed the TS-2 antigen.

The main difference between TS-1, TS-2 and CEA is that of molecular size, as determined by analytical ultra-centrifugation data. The sedimentation coefficients of the TS-1 and TS-2 antigens were 6.81 and 1.65, respectively, while that of CEA ranged from 6.9 to 8.0[11].

CEA contained a moderate amount of mannose and N-acetyl-galactosamine[11-12], while TS-2 antigen, which was absorbed with pooled blood A and B groups, had more N-acetyl-galactosamine and little mannose.

The protein parts of the TS-1 and TS-2 antigens account for 11.3% and 15.1%, respectively, and are much smaller than that of CEA, which was reported to range from 16.2% to 43.3%[13]. TS-1 and CEA have an affinity to Con A[14]. However, TS-2 antigen did not show any affinity to Con A. NCA showed cross-reactivity to TS-1 and CEA[15-16], but not to TS-2 antigen.

TS-1 and TS-2 antigens localized only on the cancer cell surfaces. They were not present on the normal cells of the lung.

In our study, TS-1 antigen was divided by proteolysis with Pronase E into TS-2 antigen and NCA. TS-2 antigen has a smaller amount of protein than CEA and TS-1 antigen, and contains no NCA. Although the purification method for TS-1 antigen is different from that of CEA, it shares a common antigenicity with CEA ; however, the antigenicity of TS-2 is different from that of CEA. TS-2 antigen purified from gastric cancer by the same method has common antigenic characteristics.

In 1979, the WHO collaborative study on lung tumor-associated antigens was held at Charing Cross Hospital. Eleven groups of workers were recruited to compare lung tumor-associated antigens extracted from tumor materials of various histological types by means of various extraction and purification methods[17]. TS-2 antigen showed crossreactivity only to antiserum of Veltri. The TS-2 antigen is different from other tumor-associated antigens except for one tumor-associated

antigen extracted by Veltri from cell membranes with Triton and separated on DEAE-cellulose[18]. Whether the TS-2 antigen is identical with that of Veltri or not remains to be determined. However, it is confirmed that TS-2 antigne is a tumor-associated antigen.

The clinical application of TS-2 antigen for the diagnosis of lung cancer has already been done.

The tumor-specific antibodies in the regional non-metastatic lymph nodes of patients with lung cancer were studied by immunofluorescent staining method. Cells with TS-2 antibody were demonstrated in 20 of 35 cases. No correlation could be established between the presence of the TS-2 antibody in the lymph nodes and stage of tumor. However, these findings are very interesting with regard to the extirpation of regional lymph nodes in the surgical treatment of lung cancer[19].

The cutaneous delaved hypersensitivity reaction was performed with TS-2 antigen in 288 patients with lung cancer to evaluate their immunocompetence levels, and it was confirmed that the response to TS-2 antigen was reduced in inverse proportion to stage advancement[20].

Further, studies concerning the measurement of TS-2 antigen in the serum of lung cancer patients by radioimmunoassay methods are still under way.

References

1) McIntire KR, Sizaret PP: Human lung tumor antigens. Excepta Medica 1: 295-299, 1974.
2) LoGerfo P: Tumor antigens associated with lung cancer. In Lung Cancer, Natural History, Prognosis and Therapy (Israel L, Chahinian AP eds). New York: Academic Press, 1976, pp. 81-93.
3) Braats JA, McIntire KR, Princler GL et al: Purification of a human lung tumor associated antigen. Fed Proc 36: 867, 1977.
4) Braatz JA, McIntire KR, Princler GL et al: Purification and characterization of a human lung tumor-associated antigen. J Natl Cancer Inst 61: 1035-1046, 1978.
5) Kempner DH, Jay MR, Stevens RH: Human lung tumor-associated antigens of 32000 daltons molecular weight. JNCI 63: 1121-1129, 1979.
6) Ibrahim AN, Rewlins D, Abdelal A et al: Tumor-associated antigens in lung cancer tissues and in sera of tumor-bearing patients. Cell Molec Biol 26: 327-333, 1980.
7) Ikeda S, Matsubara Y, Okada Y: Tumor-specific antigen in human lung cancer tissues: Purification of tumor specific antigen. (Abstract, Japanese) Proc. Jap. Cancer Assoc. of the 23nd Annual Meeting, 267, 1973.
8) Ikeda S, Matsubara Y, Okada Y: Carcinoembryonic antigen in lung cancer. (Abstract for XII International Congress on Diseases of the Chest), Chest 65: 590, 1974.
9) Winzler RJ, Devor AW, Mehl JW et al: Studies on the mucoproteins of human plasma. I. Determination and isolation. J Clin. Investigation, 27: 609-616, 1948.
10) Matsubara Y: Immunohistological study on tumor-specific antigen of human lung cancer. (Japanese) Clin. Immunol. 4: 1259-1266, 1972.
11) Krupey J, Gold P, Freedman SO: Physicochemical studies of the carcinoembryonic antigens of the human digestive system. J Exp Med 128: 387-398, 1968.
12) Banjo C, Gold P, Freedman SO et al: Immunologically active heterosaccharides of carcinoembryonic antigen of human digestive system. Nat. New Biol. 238: 183-185, 1972.
13) Banjo C, Shuster J, Gold P: Intermolecular Heterogenecity of the Carcinoembryonic Antigen. Cancer Res 34: 2114-2121, 1974.
14) David GS, Reisfeld RA: Binding of carcinoembryonic antigen (CEA) to Concanavalin A-Sepharose: Storage of high-specific-activity ^{125}I-CEA. J Nat. Cancer Inst. 53: 1005-1010, 1974.
15) Kleist S, Chavanel G, Burtin P: Identification of an antigen from normal human tissue that cross-reacts with the carcinoembryonic antigen. Proc Nat Acad Sci USA. 69: 2492-2494, 1972.
16) Neveu T, Staebler D, Chavanel G et al: Study of the antigenic cross reactivity between carcinoem-

bryonic antigen and non-specific cross reacting antigen (NCA and NCA 2). Br. J Cancer 31 : 524-527, 1975.

17) Gennings JN, Bagshawe KD, Axelsen NH et al : Collaborative study on bronchial tumor-associated antigens. Br. J Cancer 44 : 487-495, 1981.

18) Veltri RW, Mengoli HF, Maxim PE et al : Isolation and identification of human lung tumor-associated antigens. Cancer Res. 37 : 1313-1321, 1977.

19) Matsubara Y, Funatsu T, Hatakenaka R et al : Tumorspezifische Antikörper in den regionalen Lymphknoten von Patienten mit Bronchial-Karzinom. Prax. Pneumol. 34 : 524-532, 1980.

20) Ikeda S, Kuwabara M, Matsubara Y : Cutaneous delayed hypersensitivity reactions to Tumor-specific antigen in patients with lung cancer. (Abstract for 13th International Cancer Congress)' Proceedings : 479, 1982.