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A Glycoprotein Secreted by Lung Cancer Cells Is Present in Human Serum as an Immunoglobulin-binding Protein

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The 6B3·Ag recognized by a monoclonal antibody 6B3 to human large cell lung carcinoma cell line (HLC-2) is a high-molecular-weight glycoprotein of 1,000,000. Its serum level is increased in various adenocarcinoma patients. When a patient's serum with a high concentration of 6B3·Ag (54 μg/ml) or concentrated 6B3·Ag from normal human serum was analyzed by immunoelectrophoresis, 6B3·Ag showed a long bimodal precipitin line extending from the per-β to β globulin region. However, the precipitin line of 6B3·Ag in the HLC-2 culture medium was formed only in the pre-β globulin region. The 6B3·Ag was purified from pooled patients' serum by salting out, precipitation by acidification at pH 4.5 and Sepharose 4B and immunoaffinity chromatographies. Western blotting indicated that the 6B3·Ag from human serum contained IgG and/or IgM. The 6B3·Ag from human serum showed a dose-dependent reaction in a sandwich enzyme-linked immunosorbent assay with anti-6B3·Ag antibody as a solid-phase antibody and anti-human IgG or anti-human IgM antibody labeled with alkaline phosphatase. The 6B3·Ag was concluded to be partly present as a complex with IgG and/or IgM in human serum, and this complex showed a precipitin line in the β globulin region on immunoelectrophoresis.

Key words: Glycoprotein — Lung cancer cell line — Human serum — Immunoglobulin-binding

In an attempt to identify tumor markers of lung cancer cells, monoclonal antibodies (mAbs) were raised against human large cell lung carcinoma cell line (HLC-2). Among them, one antibody designated 6B3 recognized an antigen (6B3·Ag) which had been assumed to be cancer-associated in an immunohistochemical study using normal and cancer tissues (or cancer cell lines). A large quantity of 6B3·Ag was isolated from HLC-2 culture medium, purification and characterization of its physico-chemical properties were performed, and the serum level of 6B3·Ag in normal adults and patients with various diseases were also measured. All these results were reported in the previous paper,[1] in which we concluded that the 6B3·Ag is similar to L3 antigen reported by Linsley *et al.*,[2] based on a comparison of partial amino acid sequences. L3 antigen was reported to be a novel serum protein present in normal human serum at the level of about 1 to 4 μg/ml. However, the correlation between serum level and disease, and its physiological significance are still unknown. When a patient's serum with a high concentration of 6B3·Ag was analyzed by immunoelectrophoresis, 6B3·Ag showed a long bimodal precipitin line extending from the pre-β to β globulin region, while the precipitin line of 6B3·Ag from the culture medium of HLC-2 was formed only in the pre-β globulin region. Therefore, the 6B3·Ag was purified from human serum, and the reason for the formation of the bimodal precipitin line was investigated in this study.

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MATERIALS AND METHODS

Reagents Coomassie Brilliant Blue R-250 was purchased from Nakarai Chemical Ltd. (Kyoto), gelatin from Difco Laboratories (Detroit, MI), Freund's complete adjuvant from Wako Pure Chemical Industries Ltd. (Osaka), 3,3′-diaminobenzidine from Dojindo Laboratories (Kumamoto), avidin-biotin peroxidase complex kit from Vector Laboratories Inc. (Burlingame, CA), nitrocellulose membrane from Toyo Roshi Kaisha (Tokyo), Sepharose 4B from Pharmacia Chemicals AB (Uppsala, Sweden), 96-well enzyme-linked immunosorbent assay (ELISA) plates (Immunoplate II) from Nunc (Wiesbaden, Germany), human IgG and human IgM from Cappell Laboratories (Cochranville, PA), anti-human IgG antibody and anti-human IgM antibody from ATAB (Scarborough, ME), anti-human IgG and anti-human IgM labeled with alkaline phosphatase (ALP) from CALTAG Laboratories (San Francisco, CA), and anti-human serum antibody from Medical Biological Laboratories (Nagoya).

Materials The 6B3 mAb (IgG_1 subclass) was prepared using human large cell lung carcinoma cell line (HLC-2). The 6B3·Ag was purified from HLC-2 culture medium by salting out, precipitation by acidification at pH 4.5, and chromatographies on Sepharose 4B and concanavalin A (Con A)-Sepharose. New mAbs (#O-10 and #K35A3) to the purified 6B3·Ag were prepared by a standard hybridoma technique.[3,4] These procedures were described previously in detail.[1]

An antiserum to 6B3·Ag was raised in rabbits by injecting 200 μg of the antigen emulsified in Freund's complete adjuvant at intervals of a week, 4 to 5 times. The specificity of the antiserum was tested by a double immunodiffusion test[5] and immunoelectrophoresis.

Purification and characterization of 6B3·Ag from human serum Pooled human serum (500 ml) containing 6B3·Ag at 3 μg/ml was clarified by centrifugation at 10,000*g* for 30 min and fractionated by adding 180 g of sodium sulfate per liter of the serum, resulting in the formation of precipitates, which were dissolved and dialyzed in distilled water. Acetate buffer (0.5 *M*, pH 4.5) was added to the dialysates at the final concentration of 25 m*M*, resulting in the formation of precipitates, which were collected and dissolved in 10 m*M* Tris-HCl buffer, pH 8.0. The precipitation and the following solubilization procedure described above with acetate buffer were repeated twice. A part of the resulting solution was analyzed by immunoelectrophoresis, and the rest was dialyzed against buffer A (0.1 *M* acetate buffer, pH 6.0, containing 0.5 *M* NaCl) and applied to a Sepharose 4B column (2.5×90 cm) pre-equilibrated with the same buffer, followed by elution with the same buffer A. The 6B3·Ag and IgM in each fraction were detected by ELISA and double immunodiffusion test, respectively. Fractions containing 6B3·Ag were applied to a mouse immunoglobulin (Ig, mAbs which showed no reaction with 6B3·Ag)-coupled Sepharose 4B column (2.5×30 cm; 3 mg of the mAb bound per ml of the gel) to remove some Igs with affinity to the column.[6] The pass-through fraction was then applied to a #K35A3 (anti-6B3·Ag mAb)-coupled Separose 4B column (2.5×10 cm; 2.5 mg of the mAb per ml of the gel). The column was washed extensively with 10 m*M* phosphate-buffered saline, pH 7.2 (PBS), and the adherent 6B3·Ag was eluted with 3 *M* NaSCN, followed by dialysis against PBS. The concentration of 6B3·Ag was measured by ELISA using two mAbs to 6B3·Ag described previously.[1]

ELISA for 6B3·Ag complex with Igs An ELISA was employed to detect 6B3·Ag complex with IgM and/or IgG. All procedures were carried out at 37°C. Two kinds of plates, test and negative control, were used. A 96-well microplate (Immunoplate II) was coated with 100 μl of mAb (50 μg/ml in PBS) to 6B3·Ag (#O-10) for test assay or mAb which showed no reaction with 6B3·Ag, IgG and IgM for negative control assay, and incubated for 2 h. The wells were then incubated with 200 μl of 1% gelatin in PBS for 1 h to block non-specific binding before the reaction of the mAb with its specific antigen. One hundred μl of sample diluted 1:5 to 1:160 with PBS containing 10% goat serum was added to each well, and the plate was incubated for 1 h. It was washed twice with PBS, then 100 μl of anti-human IgM antibody or anti-human IgG antibody labeled with ALP was added to each well and incubation was continued for 1 h. After washing of the wells with PBS, 100 μl of substrate solution for ALP containing 4 m*M* phenylphosphate and 2 m*M* 4-aminoantipyrine in 20 m*M* carbonate buffer, pH 10.2, was added and incubated for 30 min. The reaction was stopped by adding 100 μl of 20 m*M* $NaIO_4$. The absorbance at 510 nm was measured by a microtiter plate analyzer (NJ-2000, Nippon InterMed K.K., Tokyo). The absorbance of 6B3·Ag complex with IgM and/or IgG was obtained by subtracting the absorbance of the negative control assay from that of the test assay.

Electrophoresis and western blot analysis of 6B3·Ag Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of 6B3·Ag was carried out according to the method of Laemmli.[7] The bands were stained with Coomassie Brilliant Blue R-250. Immunoelectrophoresis was carried out in 1.2% agarose gel with 75 m*M* barbital buffer (pH 8.6, μ=0.05) under a constant current of 2 mA/cm.

For western blotting, proteins on an SDS-PAGE plate were transferred electrically to nitrocellulose membrane as described by Towbin *et al.*,[8] using the electroblot system (Marysol Inc., Tokyo) at 50 V for 16 h, with cooling by circulating water. The analysis of protein bands was carried out according to the standard avidin-biotin peroxidase complex method at room temperature. The same samples were transferred to three sheets of nitrocellulose membrane (#1 to #3). The bands on the membrane were blocked to prevent non-specific binding before the reaction of the antiserum with its specific antigen by immersing membrane #1 in 10% goat serum and #2 and #3 in 10% rabbit serum for 2 h. The membranes #1, #2 and #3 were incubated with rabbit anti-6B3·Ag antiserum, goat anti-human IgG antibody and goat anti-human IgM antibody, respectively, then washed three times with PBS and the #1 membrane was incubated with biotinylated goat anti-rabbit IgG antibody, and #2 and #3 membranes were incubated with biotinylated rabbit anti-goat IgG antibody for 2 h. The membranes (#1 to #3) were then washed three times with PBS, incubated with avidin-biotin peroxidase complex for 2 h, and again washed three times with PBS. The reactive bands were visualized by immersing the membrane in 0.03% 3,3′-diaminobenzidine solution in 20 m*M* Tris-HCl, pH 7.4, containing 0.005% H_2O_2.

RESULTS

Comparison of properties of 6B3·Ag from patient's serum with the antigen from HLC-2 culture medium Antigenic comparison between 6B3·Ag from HLC-2 culture medium and a patient's serum was made by both immunoelectrophoresis and double immunodiffusion test. When the 6B3·Ag from HLC-2 culture medium and

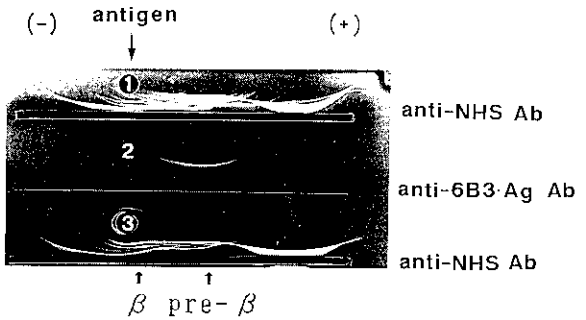

Fig. 1. Immunoelectrophoresis of 6B3·Ag from HLC-2 culture medium and in patient's serum. Normal serum, the 6B3·Ag from HLC-2 culture medium and serum of a breast carcinoma patient were analyzed by immunoelectrophoresis. Anti-NHS Ab, rabbit anti-normal human serum antibody; anti-6B3·Ag Ab, rabbit anti-6B3·Ag antiserum; 1, normal human serum; 2, 6B3·Ag (from HLC-2 culture medium, 100 μg.ml); 3, serum of a patient with breast carcinoma (6B3·Ag 54 μg/ml).

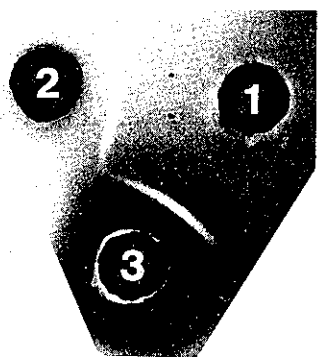

Fig. 2. Double immunodiffusion test of the 6B3·Ag from HLC-2 culture medium and in the patient's serum. 1, rabbit anti-6B3·Ag antiserum; 2, 6B3·Ag from HLC-2 culture medium (60 μg/ml); 3, serum of breast carcinoma patient (6B3·Ag 54 μg/ml).

serum of a breast carcinoma patient with a high concentration of 6B3·Ag were analyzed by immunoelectrophoresis, the precipitin line of 6B3·Ag from HLC-2 culture medium was formed in the pre-β globulin region, while the antigen in the patient's serum showed a long, bimodal precipitin line extending from the pre-β to β globulin region (Fig. 1). On double immunodiffusion, however, the precipitin lines of 6B3·Ag from HLC-2 culture medium and the patient's serum fused completely (Fig. 2). Thus, the 6B3·Ag in serum of a patient with breast carcinoma was identical immunologically to the 6B3·Ag from HLC-2 culture medium. The appearance

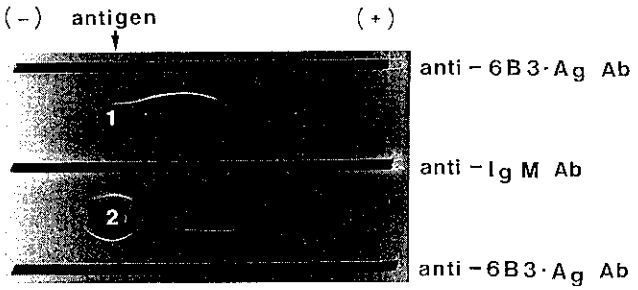

Fig. 3. Immunoelectrophoresis of 6B3·Ag from human serum. Crude 6B3·Ag fractionated by salting out and pH precipitation from pooled human serum and the 6B3·Ag from HLC-2 culture medium were analyzed by immunoelectrophoresis. Anti-6B3·Ag Ab, rabbit anti-6B3·Ag antiserum; anti-IgM Ab, goat anti-human IgM antiserum; 1, 6B3·Ag from HLC-2 culture medium; 2, crude 6B3·Ag from pooled human serum.

of a bimodal precipitin line of 6B3·Ag in the patient's serum indicated that the line in the pre-β globulin region might be due to the same 6B3·Ag as in the HLC-2 culture medium, while the line in the β globulin region might be due to 6B3·Ag aggregates, or to complex formation or interaction of 6B3·Ag with other serum components.

Analysis of properties of 6B3·Ag in pooled serum In order to elucidate the reasons for the formation of the precipitin line of 6B3·Ag from the patient's serum in the β globulin region, crude fractionation of 6B3·Ag from pooled serum containing 6B3·Ag at 3 μg/ml was attempted. The pooled patients' serum was fractionated and concentrated by salting out with 18% sodium sulfate and pH precipitation at pH 4.5, and was analyzed by immunoelectrophoresis. This fraction showed a distinct bimodal precipitin line extending from the pre-β to β globulin region with anti-6B3·Ag antiserum (Fig. 3). The same phenomenon was observed in normal adult serum pooled without purification (2.5 μg/ml of 6B3·Ag). In order to purify it further, this crude 6B3·Ag fraction was also applied to a Sepharose 4B column. The 6B3·Ag and IgM showed different peaks which partly overlapped. Both 6B3·Ag and IgM were detected over a wide range as compared with each alone (Fig. 4). Fraction B (Fr. B) and fraction M (Fr. M) were separately collected as indicated in Fig. 4. Fraction B was further purified by immunoaffinity chromatography. It was applied to a mouse Ig (mAbs which showed no reaction with 6B3·Ag)-coupled Sepharose 4B column to remove some immunoglobulins with affinity to the column. The pass-through fraction was then applied to a #K35A3 (anti-6B3·Ag mAb)-coupled Sepharose 4B column and

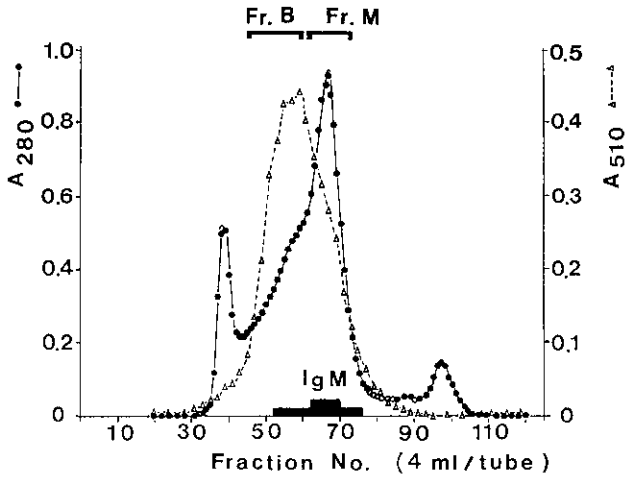

Fig. 4. Elution profile of 6B3·Ag from human serum on a Sepharose 4B column. Pooled human serum fractionated by salting out and pH precipitation as described in "Materials and Methods" was applied to a Sepharose 4B column (2.5× 90 cm) equilibrated with 0.1 *M* acetate buffer, pH 6.0, containing 0.5 *M* NaCl (buffer A) and eluted with buffer A. Closed circles indicate absorbance at 280 nm. The 6B3·Ag was detected by ELISA (△). IgM was measured by double immunodiffusion test and fractions containing IgM are indicated by the solid line.

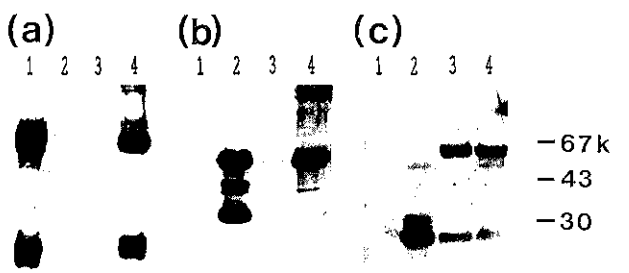

Fig. 5. Western blot analysis of 6B3·Ag from pooled human serum. Fraction B (Fig. 4) was further purified by immunoaffinity chromatography. Namely, the pass-through fraction of a mouse Ig-coupled Sepharose 4B column (2.5× 30 cm; 3 mg mAb/ml of the gel) was applied to a #K35A3 (anti-6B3·Ag mAb)-coupled Sepharose 4B column (2.5×10 cm; 2.5 mg mAb/ml of the gel). The adherent fraction was eluted with 3 *M* NaSCN, and analyzed by SDS-PAGE and western blotting as described in "Materials and Methods." Marker proteins are as follows: 67,000 (67k), bovine serum albumin; 43k, ovalbumin; 30k, bovine erythrocyte carbonic anhydrase. (a), #1 membrane; (b), #2 membrane; (c), #3 membrane; 1, 6B3·Ag from HLC-2 culture medium; 2, human IgG; 3, human IgM; 4, 6B3·Ag from pooled human serum.

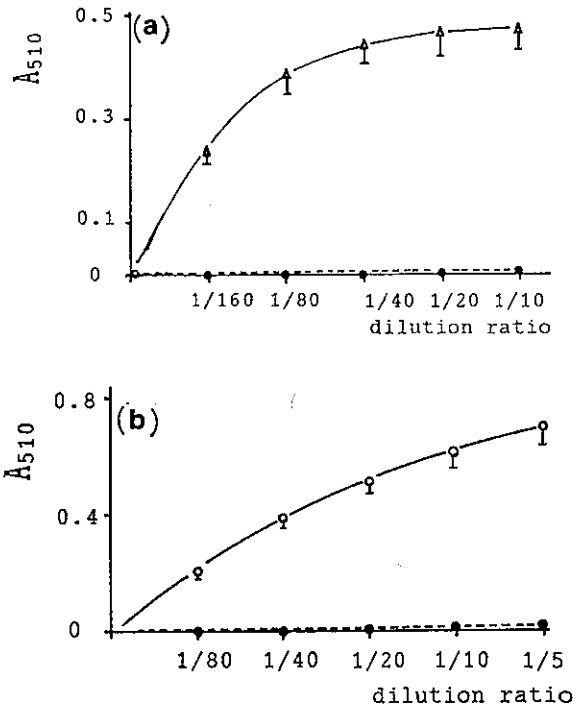

Fig. 6. The analysis of 6B3·Ag (from pooled serum) binding with IgM and/or IgG by ELISA. ELISA as described in "Materials and Methods" was performed to determine whether the 6B3·Ag from pooled serum analyzed in Fig. 5 was binding with IgM and/or IgG. Vertical bars indicate the mean − SD (N=5). (a), 6B3·Ag:IgM complex. ●, mixture of 6B3·Ag from HLC-2 culture medium and purified IgM from human serum; △, 6B3·Ag from human serum. (b), 6B3·Ag:IgG complex. ●, mixture of 6B3·Ag from HLC-2 culture medium and purified IgG from human serum; ○, 6B3·Ag from human serum.

the adherent 6B3·Ag was eluted with 3 *M* NaSCN. SDS-PAGE analysis of the 6B3·Ag fraction thus purified from pooled serum revealed four or five bands under reducing conditions by protein staining. These bands were identified as 6B3·Ag (66 and 27k), and heavy and light chain of IgG and IgM by western blotting using anti-6B3·Ag antiserum, anti-human IgG antibody or anti-human IgM antibody (Fig. 5). In order to confirm the presence of a complex of 6B3·Ag and IgM in the 6B3·Ag from pooled serum, ELISA was carried out, in which anti-6B3·Ag mAb (#O-10) as a solid-phase antibody and anti-human IgM antibody labeled with ALP were used. The 6B3·Ag from pooled serum showed a dose-dependent reaction in ELISA, and the presence of a complex of 6B3·Ag and IgM in the 6B3·Ag from pooled serum was confirmed. A simple mixture of 6B3·Ag from HLC-2 culture medium and purified IgM from

human serum (10 μg/ml) as a negative control experiment in ELISA did not show a dose-dependent reaction (Fig. 6a). In the same manner as described above, the presence of the complex of 6B3·Ag and IgG in the 6B3·Ag from pooled serum was confirmed (Fig. 6b).

DISCUSSION

When the 6B3·Ag in human serum was analyzed by immunoelectrophoresis, the precipitin line of 6B3·Ag was formed not only in the pre-β globulin region, which was the same as that of purified 6B3·Ag from HLC-2 culture medium, but also in the β globulin region. When the 6B3·Ag was purified from pooled patients' serum, and was analyzed by western blotting and ELISA, it was indicated to be partly present as a complex with IgG and/or IgM in human serum. We were interested in the possible biological significance of this finding.

It was also clarified by SDS-PAGE and western blotting that the 6B3·Ag purified partially from human serum contained 6B3·Ag, IgG and IgM (Fig. 5). Since the results in this experiment suggested the possibility of contamination with IgM or IgG, it was confirmed by sandwich ELISA using anti-6B3·Ag antibody, anti-human IgG antibody or anti-human IgM antibody that the 6B3·Ag from serum formed a complex with IgM and/or IgG (Fig. 6). Although the data are not shown, when 6B3·Ag was purified by chromatography using an anti-IgM antibody- or anti-IgG antibody-coupled column instead of the anti-6B3·Ag antibody-coupled column, the 6B3·Ag antigenicity was adsorbed on the anti-IgM antibody and anti-IgG antibody-coupled columns, indicating that 6B3·Ag in human serum is present partly as a complex with IgM and/or IgG.

The complex of 6B3·Ag with Igs was observed not only in the patients' serum but also in normal serum. The 6B3·Ag from HLC-2 culture medium was partially adsorbed on a human Igs-coupled Sepharose 4B column. As human IgG contains five sugars,[9] *N*-acetylneuraminic acid, fucose, mannose, galactose and *N*-acetylglucosamine, and the partial adsorption of 6B3·Ag on the human Igs-coupled column was not blocked by the addition of saccharides such as 0.1 *M* α-methyl-D-mannoside, α-L-fucose, *N*-acetyl-D-glucosamine, β-galactose, *N*-acetylneuraminic acid, α-D-glucose and *N*-acetylgalactosamine, the binding of 6B3·Ag to Igs is not dependent on a sugar moiety, namely, the binding is not a lectin-like reaction. The binding of 6B3·Ag to Igs was supposed to be non-covalent, since the complex was easily dissociated by addition of 0.5% SDS. It can not be ruled out that the complex of 6B3·Ag with Igs is based on antigen-antibody reaction. The complex has functions of an immune complex, such as antigen exclusion, complement activation and others. Further investigations are necessary.

A cell-binding immunoglobulin-like protein which has very similar properties to those of 6B3·Ag was reported by Ohori *et al.*[10,11] They called it CIP, and isolated it from normal human plasma by adsorption on and elution from an elastin-Sepharose column, and its molecular structure was studied by SDS-PAGE and immunoblotting. Its molecular weight was reported to be more than 900,000. However, since CIP provided by Fujimoto did not react with anti-6B3·Ag antiserum, it was concluded that the 6B3·Ag is distinct from CIP.

Accurate determination of the ratio of free 6B3·Ag to 6B3·Ag complex with Igs was not carried out, but the ratio was estimated to be about 30 to 40% in serum of a patient with a high concentration of 6B3·Ag, judging from the immunoelectrophoresis pattern (Fig. 1). The steps of concentration by salting out and pH precipitation used to purify 6B3·Ag from normal human serum may alter the ratio of free 6B3·Ag to 6B3·Ag complex with Igs. Nevertheless, the ratio in normal serum was estimated to be 50% or more 50%, which was the same as or higher than that of patients' serum (Fig. 3).

The physiological significance of 6B3·Ag is unknown at present. However, some interesting results were obtained in a preliminary study. The 6B3·Ag was present in melanoma, lung cancer cells and various adenocarcinoma tissues as a shedding type,[2,12] and its serum level was found to be elevated in patients with malignant diseases.[1] It is noteworthy that the 6B3·Ag exhibited a specific cytotoxic activity toward hybridoma cells (anti-6B3·Ag, anti-α-fetoprotein, anti-carcinoembryonic antigen or anti-C-reactive protein mAb-producing cells) *in vitro* at 5 μg/ml. It is also interesting that the 6B3·Ag remarkably suppressed the growth of mitogen (lipopolysaccharides)-induced mouse lymphocytes *in vitro*. It was supposed from these results that the 6B3·Ag might be partially responsible for the reduced immune functions of patients with malignant neoplasia. Further studies are in progress. Examination of the ratio of free 6B3·Ag to Ig-associated 6B3·Ag in various diseases and its binding mechanisms with Igs should help to clarify the biological activity of 6B3·Ag.

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