Identification and Purification of a Human Lung Tumor-associated Antigen from a Primary Lung Tumor

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ABSTRACT

A human lung tumor-associated protein has been purified from an extract of a human small cell carcinoma of the lung and shown by Ouchterlony double diffusion analysis to be antigenically identical to a component which was previously demonstrated in 84 of 98 lung tumor extracts of all histological types but absent from extracts of normal adult and fetal lung, other normal tissues, and tumors of other organs. These studies utilized xenon antisera raised against a pool of lung tumor extracts which were exhaustively adsorbed with normal serum and tissue extracts. A radial immunodiffusion assay developed for the antigen permitted its quantitation throughout the course of isolation. Purification was accomplished by ion-exchange chromatography, gel filtration, and affinity immunoadsorption. By ion-exchange chromatography, the proteins appeared to be quite heterogeneous, with immunological reactivity detected in three different peaks. However, all the active components were immunologically identical. Gel filtration of the major antigenic component from diethylaminoethyl cellulose similarly demonstrated a further fractionation into several active, immunologically identical forms. These results suggest a charge-size isomeric relationship among the various forms, all of which possess a common and identical antigenic site. The major component was isolated throughout the purification scheme. The final product represented 9% of the input activity, produced a single, although broad, protein-staining region on 7% polyacrylamide gels which was coincident with antigenic activity, and exhibited immunological identity with the antigen in the crude extract as well as with that in an extract from another lung tumor.

INTRODUCTION

A great deal of effort has been expended in recent years in a search for human tumor-associated antigens, especially those that might be selective for a particular organ site (10). Our laboratory has been primarily interested in the study of human LTA2, and other groups have also identified a variety of human lung tumor-related products (1, 7, 11–13, 16, 18–21). The goals of most of these studies are to purify and characterize, from extracts of 2 different lung tumors, of similar tumor-associated antigens that reacted, after radioiodination, with immune serum (2, 8). For one of these, LTA-2b, a radioimmunoassay was developed which appeared to be able to discriminate, by quantitation of serum values, a high percentage of patients with active lung cancer from those with lung cancer in remission following complete surgical resection (15). However, the basal values of the lung tumor antigen in normal individuals were higher than expected. The cause of the high basal level was later found to be due to inhibition of binding in the radioimmunoassay by serum ACT. Despite this competition in a double-antibody radioimmunoassay, LTA-2b and the ACT were physicochemically different glycoproteins (9), and the LTA-2b showed no inhibitory activity toward chymotrypsin (9).

This ACT-related lung tumor-associated antigen appears to be distinct from the antigen originally identified by Ouchterlony analysis. The final stages of purification were monitored by antibody precipitation of radioiodinated antigen-containing fractions in place of the double-diffusion assay in order to conserve purified antigen. It later became evident that the 2 assays were measuring distinct antigenic activities, probably due to the large difference in concentration of antiserum. This situation would favor antigen binding with high-affinity antibodies in the radioimmunoassay at low antiserum concentration and binding with low-affinity antibodies in the double-diffusion assay at high antiserum concentration. In order to assure the isolation of the antigen demonstrated by double-diffusion analysis, we developed a quantitative assay based on a precipitation-in-gel reaction, which more closely approximates the Ouchterlony analysis. The description of this assay and its use in the isolation of the common LTA, forms the basis of this report. Partial characterization of the antigen and the development and application of a first generation radioimmunoassay are described in the accompanying paper (3).

MATERIALS AND METHODS

Materials. Cyanogen bromide-activated Sepharose 4B, Protein A-Sepharose, and Sephadex G-200 were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N. J., and DEAE-cellulose was obtained from Whatman, Inc., Clifton, N. J. Agarose was obtained from L'Industrie Biologique Française, S. A., Gennevilliers, France. All chemicals were the best grades commercially available.
Lung Tumor Extraction. Primary tumor and metastasis to the liver, from a patient with small cell carcinoma of the lung, were combined and had a total weight of 1657 g. This pool of tissue is referred to here as LT-120. The tissue was cut into 0.5-inch cubes and divided into 8 portions, and 275 ml phosphate-buffered saline (0.01 M sodium phosphate, pH 7.4, containing 0.15 M NaCl) were added to each. The tissue was disrupted by two 30-sec full-power bursts in a Brinkman Polytron homogenizer, using a PT-35 generator. The homogenate was centrifuged for 1 hr at 16,300 x g in a Sorvall GSA rotor. The supernatants were poured through a gauze filter, combined, and stored at -20°C.

Antiserum. Rabbit antiserum, designated R-152 and R-201, have been described previously (2). All tests by double diffusion in gel have indicated that they detect the same antigenic component. These adsorbed antiserum detected a protein in 84 of 98 lung tumor extracts and in 1 of 13 extracts of tumors from organs other than lung but were negative, at the level of sensitivity of the double-immunodiffusion test, with normal lung extracts, normal tissues, fetal tissues, and normal serum.

LTA Assay. To quantitate human LTA, we used a radial immunodiffusion test similar to those described by Mancini et al. (14) and Fahey and McElvee (6). The IgG fraction from rabbit antiserum R-152 was isolated by precipitation with 45% (NH4)2SO4, followed by adsorption to Protein A-Sepharose and elution with 0.1 M glycine-HCl, pH 3.0. After the pH was adjusted to 7.0 with 0.5 M Na2CO3, the volume of the solution was brought to the original volume of the antiserum used for the isolation. A 1:25 dilution of this purified IgG in 0.05 M Veronal buffer, pH 8.6, at 35–40°C, was mixed with an equal volume of 2% agarose in the same buffer at 75°C. Of this mixture, 8.5 ml were applied to a 5- x 7.5-cm glass slide (previously coated with 0.3% agarose and dried), kept in a moist chamber at 4°C, and allowed to gel. Wells (1.3 mm) were punched in the agarose, and samples (approximately 3 μl) were added in duplicate. Serial dilutions of a lung tumor extract, LT-37, were used as standards, and a unit was arbitrarily defined as the amount of antigen required to produce a circular precipitin ring 2.7 mm in diameter. The plates were incubated under light paraffin oil for 42 hr at 37°C, rinsed, and washed overnight in phosphate-buffered saline. The gel surface was covered with filter paper, dried at 37°C, stained for 10 to 15 min with 0.1% Amido Black, and then destained with 5% acetic acid until a clear background was obtained. The diameters of the precipitin rings were measured, and the calculated areas were plotted against the concentration of standard as shown in Chart 1. The assay was linear over a range of 1 to 30 units.

ACT Assay. A radial immunodiffusion assay, similar to the radial immunodiffusion assay for LTA, was performed as described previously (17). This assay has a range of 5 to 200 μg of ACT.

Double-Diffusion Analysis in Agarose. Ouchterlony double diffusion analysis was performed in 1% agarose gel for 24 to 48 hr at 20°C as described previously (2).

DEAE-Cellulose Chromatography. A column of DE-52, 350 ml bed volume, was equilibrated with 5 mM Tris-HCl, pH 8.1 (Buffer A), at 4°C until the effluent had the same conductance as the starting buffer. The crude extract was dialyzed against Buffer A containing 0.5 mM phenylmethylsulfonyl fluoride and 0.02% NaN3 and then loaded onto the column which was washed with Buffer A until the absorbance of the effluent at 280 nm was less than 0.07. A linear NaCl gradient from 0 to 0.2 M, with a total volume of 4 liters, was applied to the column, followed by a 1 M NaCl wash. All NaCl solutions were prepared in Buffer A. Portions of fractions were concentrated using a Minicon type B15 concentrator (Amicon Corp., Lexington, Mass.) and tested for LTA or ACT content. Fractions eluting with the NaCl gradient that contained antigenic activity were pooled as indicated and then concentrated by ultrafiltration with an Amicon concentrator and a UM-20 membrane (Amicon) to a final volume of 35 ml.

Gel Filtration. A column of Sephadex G-200, 3.5 x 143 cm, was equilibrated with 50 mM Tris-HCl, pH 8.1, at a flow rate of 15 ml/hr. The column was calibrated with blue dextran and chymotrypsinogen A (Sigma Chemical Co., St. Louis, Mo.), bovine serum albumin (Miles Laboratories Inc., Elkhart, Ind.), and NaN3.

Affinity Chromatography. The IgG fraction from rabbit antiserum R-152 (25.8 mg, prepared as described under "LTA Assay") was coupled to CNBr-Sepharose (35 ml gel) using the manufacturer's recommended procedure. Pooled fractions of antigenic activity from the G-200 profile were passed through the affinity column at 4°C. The column was washed with the equilibrating buffer (0.01 M Tris-HCl, pH 8.1, containing 0.5 M NaCl) and then eluted with 2.5 ml NH4SCN, pH 8.0. Fractions which eluted with thiocyanate were immediately pooled, dialyzed against the equilibrating buffer, and stored at -20°C.

Gel Electrophoresis. Analytical polyacrylamide gel electrophoresis was performed according to the method of Davis (5) without stacking gels. Staining for protein was performed using Coomassie Brilliant Blue G-250 as detailed in Ref. 2. For the determination of antigenic activity
in the gel after electrophoresis, 2.5-mm slices were placed in agarose wells of the same diameter, and double-diffusion analysis against rabbit antiserum R-201 was performed.

Analytical Procedures. Protein was determined with the Bio-Rad protein assay reagent, using bovine serum albumin as standard (4).

RESULTS

The crude extract from tumor tissue (about 2 liters) was subjected to DEAE-cellulose chromatography. The elution profile indicated multiple peaks of antigenic reactivity (Chart 2). Three major regions containing antigenic activity are referred to as Peaks I, II, and III in their order of elution. A negligible amount of activity, Peak IV, eluted with 1 M NaCl. The predominant component, Peak II, eluted soon after the application of the gradient, at about 0.045 M NaCl, and contained more than 4 times as much antigen as did Peak III, which eluted at about 0.08 M NaCl. Peak I contained almost one-fourth as much activity as did Peak II but did not result from overloading the column; when it was reapplied to a fresh column of DEAE-cellulose, three-fourths of the antigenic activity was again recovered in the unbound fractions. Despite the fractionation of antigenic activity into multiple components, we observed the antigens in Peaks I, II, and III to give precipitin lines of identity by double-diffusion analysis (Fig. 1). In addition, each of the 3 components produced lines of identity with our standard lung tumor extract, LT-37, which itself is representative of most lung tumor extracts which have been tested in this fashion. There-fore, these components appeared to have identical antigenic sites, which were also common with human lung cancers in general.

The quantitative aspects of this and the subsequent purification steps are presented in Table 1. The sum of the activity present in the 4 peaks represents 75% of the activity applied to the column, while Peak II alone accounted for 49% of the activity.

Fractions were also assayed for ACT, since we have previously reported on the isolation of LTA-2b, which is immunochemically related to ACT. Peak II was clearly separated from ACT and thus, by this analysis, was distinct from LTA-2b. Peak III showed some overlap with ACT; however, the 2 peaks appeared distinct. Based on additional evidence to be presented below, they seem to be unrelated.

Concentration of Peak II by ultrafiltration followed by Sephadex G-200 chromatography resolved the antigenic activity into 2 additional components (Chart 3). The major peak eluted at a Ve/Vo of 1.4, corresponding to globular proteins with molecular weights of 135,000 to 150,000. Approximately 41% of the applied activity was contained in this pool of fractions, whereas the minor peak eluted at Ve/Vo of 1.75 and contained about 10% of the applied activity. The major peak may represent a more polymerized form of the minor peak, since the latter eluted in a region containing globular proteins with molecular weights of approximately 80,000, roughly one-half the size of the major component. The 2 forms were immunochemically related, giving a precipitin line of identity in Ouchterlony double diffusion (Fig. 2). Each peak was further purified by affinity immunoadsorption chromatography.

The IgG fraction isolated from the rabbit immune serum (R-152) was coupled to Sepharose 4B and used as the affinity
Polyacrylamide gel electrophoretic analysis of the fraction which bound to and was eluted from the antibody column demonstrated the degree of purity of this material. Fig. 3 shows a representative pattern obtained when the affinity-purified preparation was electrophoresed on a 7% acrylamide gel. One major protein-staining component was evident, with some diffuse anodal staining. For the purpose of locating the antigenic reactivity, an identical gel with the same protein load (27 μg) was electrophoresed at the same time. The tracking dye migrated to the same position in both gels, 50 mm. Slices (2.5 mm) of the unstained gel were tested for immunoprecipitable reactivity, an identical gel with the same protein load (27 μg) was electrophoresed at the same time. DEAE Peak II. In addition to this, Peak III from DEAE-cellulose chromatography of DEAE Pool III. Fractions were tested for absorbance at 280 nm (λ), LTA (+), and ACT (3) activity by radial immunodiffusion. Two pools of activity were collected: Pool C, 549 to 905 ml; Pool D, 913 to 1171 ml. Standards are as indicated in Chart 3.

Affinity immunoadsorption was also performed on Peak B (Chart 3) and Peak C (Chart 4). When these pools of affinity-purified antigens (Affinity Pools 2 and 3, respectively) along with the first affinity-isolated antigen (Affinity Pool 1) were tested in double diffusion, lines of identity resulted not only among each other but also with the antigen present in a standard extract, LT-37 (Fig. 4), indicating the common nature of these antigens. Lines of identity between the purified and crude forms of the antigens were also demonstrated (Fig. 4, Wells 1 and 2), suggesting that the antigen was isolated intact.

**DISCUSSION**

Our previous attempts at isolating antigens from crude lung tumor extracts were dependent upon reactivity of partially purified and radiolabeled antigens with high dilutions of antisera (2, 8). By this analysis, several proteins were isolated from extracts which, after radioiodination, would bind to immune but not to control rabbit sera. It was difficult to decide which of these antigenic components were related to the antigen detected by Ouchterlony analysis with the same immune serum. In the radioimmunoprecipitation assay, the antiserum was used at a much higher dilution than in the double-diffusion analysis. Thus, higher-affinity antibodies might account for the reactivity in radioimmunassay, whereas lower-affinity antibodies within the same antiserum might be detecting an unrelated antigenic component in the Ouchterlony tests. To assure the isolation of the original antigen detected by the precipitation-in-gel procedure, we used a quantitative modification of the same method to monitor each step of the purification.

The final purified preparation, which represented the major active component throughout the isolation, contained 9% of the activity present in the crude extract. This material produced one low-mobility component on a 7% polyacrylamide gel, which comigrated with the antigenic activity. The relatively low recovery is partially attributable to the subdivision of activity at various steps of purification. The total activity recovered during the affinity immunoadsorption step was 20% of that in the crude extract, but even this does not take everything into account since, for example, DEAE Peak I was not processed through the affinity column. The overall recoveries at each step, based on the activity applied at each of those steps, were quite good. Thus, recoveries of 75, 50, and 68% were obtained from DEAE-cellulose, gel filtration, and immunoadsorption, respectively. At each step, the various peaks of activity produced lines of identity in Ouchterlony analysis. Nevertheless, these forms were kept separate, and only the major component in each step was considered for characterization studies. They may simply represent aggregation products and/or the presence of charge isomers. Heterogeneity of this type may be a problem intrinsic to tumor extracts and to the plethora of degradative enzymes released during necrosis which might modify the structure of these components. We are currently studying the production of this antigen by lung cancer tissue culture cell lines and hope to shed some light on this question.

The antiserum used in these studies forms precipitin lines of identity in 86% of the 98 lung tumor extracts examined. The ubiquity of this antigen could render it a useful tumor marker. Quantitation of proteins of this type in patients' sera by a sensitive, specific assay is a major effort in many laboratories concerned with immunodiagnosis of malignant disease. Our studies in this regard concerning the antigen described in this report, along with its partial characterization, are the subject of the companion paper (3).
REFERENCES

Fig. 1. Ouchterlony double-diffusion analysis of DEAE-purified antigens. Center well, adsorbed immune serum R-152; Well 1, Pool I; Well 2, Pool II; Wells 3 and 6, LT-37; Well 4, Pool III; Well 5, Pool IV. The minor reactivities seen with Wells 1, 4, and 5 are residual normal reactivities due to incomplete adsorption and are not related to the major tumor antigenic component.

Fig. 2. Ouchterlony double-diffusion analysis of G-200-purified antigens. Center well, adsorbed immune serum R-201; Wells 2, 4, and 6, LT-37; Well 1, Pool A (from Chart 3); Well 3, Pool B (from Chart 3); Well 5, Pool C (from Chart 4).

Fig. 3. Polyacrylamide gel electrophoresis of affinity-purified LTA and detection of antigenic activity. Two 7% polyacrylamide gels, each containing 27 µg protein, were electrophoresed; one was sliced into 2.5-mm sections; and the other was stained with Coomassie Blue. Each slice was placed in an appropriately sized well, and the protein was allowed to diffuse against R-201 (center well). Upper and lower wells (unlabeled) in each pattern contained LT-37 for comparison. Slices 9 to 24 were all negative for antigen.

Fig. 4. Ouchterlony double-diffusion analysis of affinity-purified LTA. Center well, adsorbed immune serum R-201; Well 1, crude extract; Well 2, Affinity Pool 1; Wells 3 and 6, LT-37; Well 4, Affinity Pool 2; Well 5, Affinity Pool 3.
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