ISOLATION AND CHARACTERIZATION OF A HUMAN LUNG TUMOR ASSOCIATED ANTIGEN[+]

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Abstract

This research is designed for isolation, purification and idenification of human lung tumor associated antigen from two different tumors using perchloric acid, affinity chromatography, polyacrylamide gel electro-phoresis and sucrose gradient sedimentation analysis. The lung tumors have been shown to have identical physicochemical properties and appear to be composed of three subunits, each with a molecular weight of 25000 .

المستخلص

تم في هذا البحث عزل وتنقية وتوصيف المستضد الورمي الرئوي من انموذجين ورمين مختلفين من اورام الرئة باستخدام عدة تقنيات شملت الاستخلاص بحامض البركلوريك، كروماتوغرافيا الالفة، الترحيل الكهربائي بهلام الاكريلامايد والترسيب المتدرج باستخدام السكروز. اظهر المستضد المعزول من الاورام المختلفة صفات فيزوكيميائية متماثله كما اظهرت تجارب التوصيف ان هذه المستضدات تتألف من ثلاث وحدات كل منها ذات وزن جزئيي ٢٥٠٠٠ كيلو دالتون.

Introduction

There have been a number of reports concerning the identification and isolation of human lung tumor associated antigens[1,3]. Our laboratory has become interested in this problem as first step in the development of an immunodiagnostic test which could find application in early diagnosis or in monitoring therapy in lung cancer patients. The availability of a purified, homogeneous, well characterized lung tumor associated antigen should lead directly to the development of a radio immunoassay which could then be assessed for its value in diagnosis and¯ prognosis.

The approach to this problem has been to develop an antiserum capable of detecting an antigen in lung tumor extracts which cannot be demonstrated in normal tissues. This antiserum is then used to isolate the antigen by immunoadsorption chromatography[4].

Chemicals

All chemicals and reagent used in this study were of analytical grade.

chemicals	Company
Tris (hydroxymethyl) amino methan, apo-ferritin, catalas, alkaline phosphatase, bovin	Fluka: Switzerland

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serum albumin, ovalbumin, chymotryp sinogen, myoglobin, cytochrom c sodium dodecyl sulfate, Ammonium persulphate Ethand, glacial acetic acid	
NaN_3, NaCl, NH_4SCN Perchloric acid coomassie Brillant Blue R_{250}	BDH, limited, Poole (UK)
Sephadx G-200, CNBR- activated Sepharose acrylamide N,N- methylene bisacrylamid	Pharmacia Fine Chemicals (Sweden)
Chloramines . T	Sigma

Instruments

Instruments	Company
1-Gamma counter type 120- rack Gamma Iia, 2- Electrophoresis	LKB
UV- 210 a double beam spectrophotometer	Shimadzu
PH- meter	Pye-Unicam
Cooling centrifuge	Hettich
Cooling centrifuge type 202 MK	Sigma
SM- shaker	England

Patients and Specimens

The tumor tissues were surgically removed from lung tumor patients. Specimens were immersed in ice-cold isotonic saline solution.

They were collected individually in plastic receptacle and stored at $-20C^0$ until homogenization.

Malignant tumor (5 Males) who subjected to curativ surgery. Their age mean 44 ranged (35-70 years).

The patients were admitted for treatment to nursing Home Private Hospital.Antisera Pooled saline extracts of five primary lung carcinomas were treated with 0.5 M of perchloric acid and the soluble antigens, after neutralization by dialysis, were used as immunogens. These procedures have been detailed elsewhere [5].

Mthods

1-Affinity chromatography

The IgG fraction (105mg) from the immune rabbit serum was coupled to CNBR- activated Sepharose 4B (25ml) according to the manufacturer's recommended procedure[5]. After equilibration in a column with buffer A (which contained from 5m M Tris, 0.002% NaN_3. pH 8.1), a 100.000xg supernatant(1200 mg protein in 22.7 ml) of a human lung adenocarcinoma designated(LT-110) was passed through, washed with(1) buffer A, (2) buffer A plus 0.5M

NaCl. And[3]2.5M NH_4SCN pH 8.0. Fractions were pooled, dialyzed, concentrated and tested in double diffusion against immune serum. The proteins eluted with thiocyanate were radioiodinated using chloramines T as described[6].

2-Preparative gel electrphoresis

As a second step in purification, proteins from affinity chromatography were separated by preparative polyacrylamide gel electrophoresis(PAGE) and eluted from the sllees by revesed electrophoresis as previously described[7].

3-Gel filtration

For analytical work a 1.5 x 60 cm sephadex G-200 column was equilibrated with 0.05 M Tris, 0.002% NaN_3, 0.5 M NaCl, pH 8.1. Fractions (0.85ml)were collected at a flow rate of 2.9 ml per hour. For preparative work a 1.5 x 95 cm sephadex G-200 column equilibrated with 0.05 M Tris, 0.1 M NaCl, pH 8.1, was used at a flow rate of sml/hr; 1ml fractions were collected.

4-Analytical gel electrophoresis

Non-detergent gels were run according to [7] except stacking gels were omitted and sodium dodecyl sulfate(SDS) gels were as described by [7].

5-Two-dimensional immoelectrophoresis

Proteins were electrophoresed in a 7% polyacrylamide gel which was subsequently set on a glass plate layered with 1% agarose in 0.025M Veronal buffer , pH 8.6, containing immune serum at a dilution of 1:50. Forty volts were applied across the 3.25 inch length of plate for 2 hr. the plate was then washed overnight in buffer, dried and stained for protein by coomassie Brillant Blue R-250.

6-Analytical procedures

Protein was determined by method of Lowry et al[9]. Using BSA standard. Double immuno diffusion was performed in 1% agarose for 24-48 hr at $37c^o$, dried and stained with Amido Schwartz. Antibody radioimmunoprecipitation was performed by incubating ^{125} I-antigen with control or immune rabbit serum in the presence of BSA for 18 hr at $4c^o$. the free and Ig-bound antigen were separated by precipitation with 50% ammonium sulfate.

Results

The first step in purification of the lung tumor associated antigen (LTAA) was affinity chromatography using the IgG fraction from the rabbit tumor – immune serum. Fig (1) :show a typtical profile obtained

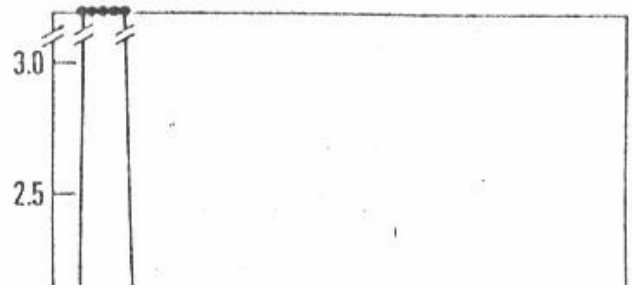
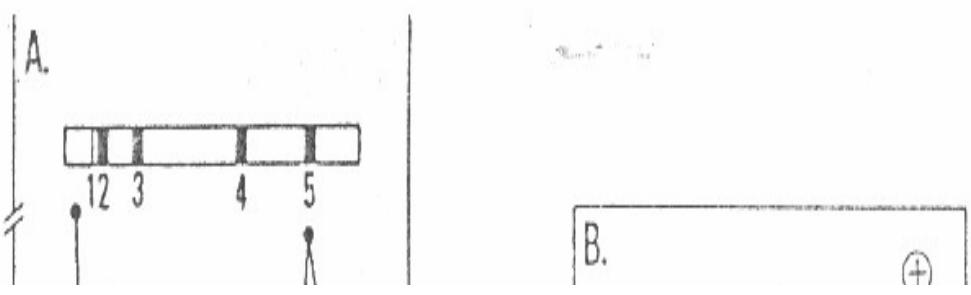


Fig (1) : Immunoadsorption chromatography of a soluble saline extract of a human lung adenocarcinoma (LT-110)

Tumor extract passed through a column of immunoadsorbent. Antigen was eluted from the column with 2.5M NH_4SCN and could not be detected in the preceding elutions. Although specific activities could not be quantitatively assessed, it was obvious from the reactivity patterns by double diffusion that the thiocyanate eluate was considerably enriched with tumor antigen.Thus, compared to the original extract, the thiocyanate eluate produced a much more intense precipitin line at one – twentieth the protein concentration.

Polyacylamide gel analysis of region III(Fig.1) revealed at least five components. This mixture of proteins was radioiodinated to facilitate monitoring during subsequent isolation procedures. Fig. 2B shows the acrylamide gel profiles of labeled and unlabeled protein. In order to determine which component was the precipitating antigen, another portion of the material isolated by affinity chromatography was subjected to two-dimensional immunoelectrophoresisi as shown in Fig. 1B. only one"roket" of precipitation was evident, and this corresponded to the fourth protein band from the origin. This will be referred to as LTAA-2a. Isolation of each of the radioiodinated components by preparative gel electrophoresis and subsequent binding studies with immune serum also supported the conclusion that LTAA-2a was the antigenically active component. This was based on the finding that it reacted to a greater extent than did the other components although some radioprecipitation was detected in bands 2 and 3 as wel[4].



Since LTAA-2a appeared to be the antigenically active component, it was subjected to further characterization after isolation by preparative gel electrophoresis. Fig. 3 shows 6 the purified and radioidinated antigen after electrophoresis on (7%) acrylamide gel. Only one component was observed; furthermore, PAGE at different acrylamide concentrations all showed a single peak of radioactivity.

Molecular sieve chromatography of I-LTAA-2a produced a single peak which, when compared to the elution of standard globular proteins, had a molecular weight of 108,000(Fig.4). Howver, when subjected to SDS gel electrophoresis, a single component with a molecular weight of 42,000 was obtained which varied by less than (5%) on gel of 7.5, 10.0, and (12.5%) acrylamide. The lack of an integral relationship between these two values prompted further examination of the labeled antigen.The molecular weight of LTAA-2a was calculated using the Svedberg equation. In order to do this the sedimentation and diffusion coefficients had to be experimentally derived.

The former was determined by velocity sedimentation in a.(5-20%) sucrose gradient according to [10]. A value of 4.24S was calculated by comparison to two internal protein standards.

The diffusion coefficient was calculated from the sephadex gel chromatogrophy data whereby the $D_{20,w}$ was interpolated from a plot of the reciprocal of the diffusion coefficient vs the elution to void volume ratios[11] and was found to be 5.0×10^{-7} cm^2 Sec^{-1}. these values , in conjunction with an assumed partial specific volume ($\bar{v}$) of 0.735 cm^3 gm^{-1}, gave a molecular weight of 77.000. Although this value is closer than the 108.000 value to being integrally related to the subunit size of 42.000 (1.83 vs 2.57), it has to be considered as tentative pending an experimental value for the($\bar{v}$). however, as an independent check on the molecular weight, the antigen along with standard proteins was subjected to polyacrylamide gel electrophorsis at varying concentrations of acrylamide according to [12]. Plots of relative

mobility vs acrylamide concentration yielded straight lines for each protein, the slopes of which in secondary plots were proportional to the molecular weight. From these data a value of 73.000 was obtained for LTAA-2a which closely agrees with the value obtained by using the Svedberg equation. The assumed value for ($\bar{v}$)therefore appears reasonable.

The data obtained from G-200 chromatography of labeled antigen and standard proteins were also used to calculate the Stokes radius (a) of the antigen. Plotting $(-\log K_{av})^{1/2}$ vs (a) produces a straight line[12] from which a Stokes Radius of $40A^{o}$ was obtained.

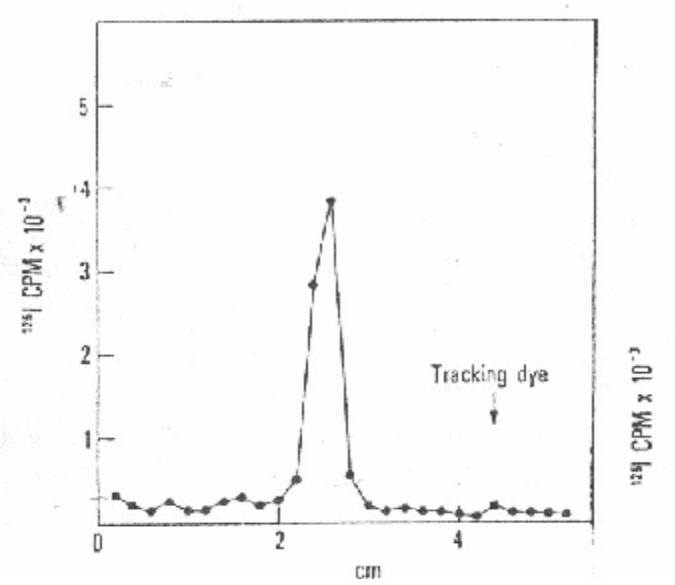


Fig (3): Polyacrylamide gel electrophoresls of purified LTAA-2a.

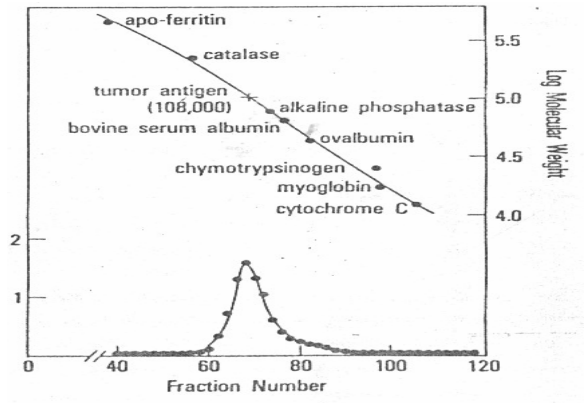


Fig.(4) :G-200 chromatograph of purified LTAA-2a and standard globular proteins for Determination of M. Wt by gel filtration chromatography

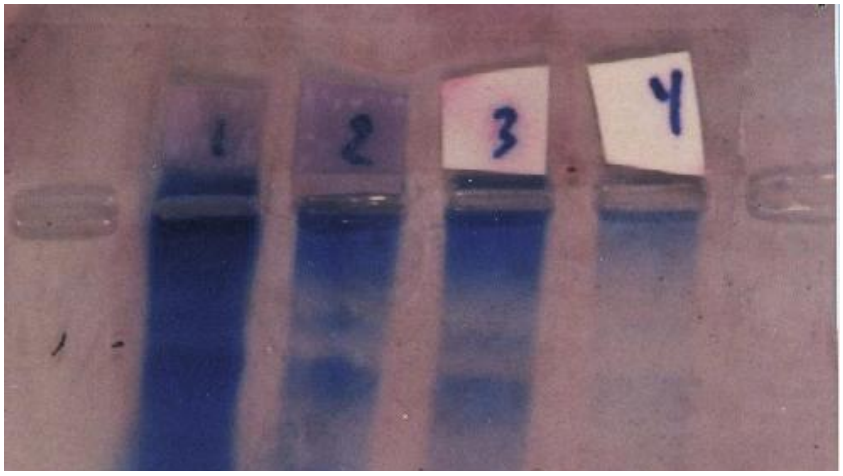
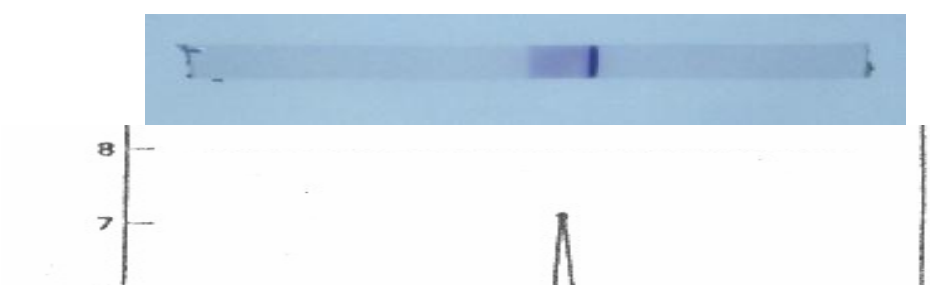


Fig. (5): Polyacrylamide gel electroplhoresis of antigen-containing fractions from LT-105 after various steps of purification . 1. After DEAE- cellulose.2. After G-200 chromatography. 3. After the first preparative PAGE .4. After the second preparative PAGE.

This value was then used to calaulate a value of 1.42 for the frictional ratio which indicates some degree of asymmetry to the molecule, or hydration, or both. This asymmetry could explain the anomalously high value for the molecular weight obtained by using gel chromatography .

Previous serological studies[16] had demonstrated this LTAA in different tumor extracts and exhibited lines of identity using the immune rabbit serum. In an attempt to confirm this observation and at the same time isolate larger quantities of the antigen, another lung tumor extract was utilized, LT 105, for the isolation of the LTAA. To assist in the isolation, a small amount of purified and radiolabeled LTAA-2a from LT-110 was added to the crude extract of LT-105, but at the same time, precipitating activity to coincide throughout the purification procedure[5]. The purification scheme consisted of DEAE- cellulose chromatography, sephadex gel filtration and preparative PAGE. The overall recovery of labeled antigen was (9%). Fig (5) shows acrylamide gel profiles after each step of purification. The final product appeared homogeneous and, after radioiodination, both labeled and unlabeled species co-migrated as a single component (Fig.6). This radiolabeled protein(designated LTAA-2b) was then subjected to all the physical-chemical analyses described above for the first antigen. All physical properties were found to be the same within experimental error and are tabulated and compared in table 1. In addition to these studies, both antigens were chromatographed on Biogel A-5M in the presence of 6M guanidins hydrochloride and 0.1M DTT[13].



Under these conditions, the antigens were denatured to polypeptides with the native values of 73.000 and 77.000 for LTAA-2a and 76.000 for LTAA-2b than do the values from SDS gels of 42.000 and 46.000 for LTAA-2a and LTAA-2b, respectivelys Since glycoproteins are known to exhibit erroneous value in SDS gels due to diminished binding of detergen[14].

In one experiment a partially purified preparation from LT-105. used,was greatly enriched in the LTAA. This was electrophoresed in SDS gels and stained with periodate-Schiff's reagent (PAS). The LTAA, as identified by trace amounts of radiolabel, stained well with PAS. In anothour set of experiments the purified antigens from both tumors were treated with neutaminidase. In both cases the treated antigens displayed decreased electrophoretic mobility(to the same extent) on (7%) acrylamide gels. Furthermore, while both antigens were eluted as single peaks from DEAE-cellulose with 0.15M NaCl, both neuraminidase-treated froms eluted as single peaks with 0.10M NaCl.

The result tabulated in table (1) reveal that the human lung tumor associated antigen isolated from two different lung tumors has been shown to have identical physiochemical properties.The purified antigens were homogeneous disc gel electrophoresis in the presence and absence of detergent at several concentrations of acrylamide, gel filtration, ion exchange chromatography and sucrose gradient sedimentation analysis. The antigen appears to be composed of three subunits. Each was with a molecular weight of 25.000 [14].

A frictional ratio of 1.4 was calaulated which indicates that the shape of the molecule may deviate from a sphere. Alternatively, it could be an indication of hydration[15]. If the antigen is asymmetric, it would elute from gel filtration columns with globular proteins of higher molecular weight. Thus the values of 98.000 and 108.000 would be overestimates.

That the same antigen was isolated from two distinct tumors is supported by their similar esdimentation and diffusion coefficients, stokes radii, frictional ratios, and subunit and native

molecular weights. Both antigens behave similay on DEAE-cellulose and have similar R_fs in acrylamide gels. Both are affected to the same extent by treatment with neuraminidase and are equally reactive with the tumor immune serum. Mixtures of the two radiolabeled antigens could not be separated by acrylamide gel electrophoresis or Sephadex gel chromatography. In fact, the only difference noted during the course of these studies is their behavior in SDS electrophoresis at low concentration of acrylamide. The antigen from LT-105 shows a variation in the observed molecular weight between 5 and (12.2%) acrylamide whereas the LT-110 antigen was invariant between 7.5 and (12.5%). This could be reflection of microheterogeneity in the carbohydrate protein of the molecule to differ significanity in other respects such as size, shape, charge, etc.

These studies indicate that the same (or very similar) molecular entity, defined both serolgically and biochemically, was present in two different human lung tumors and based on the serological data, may be common to large percentage of these tumors. This fact satifies one criterion which must be met in order for an immunodia-agnostic test to be feasible, namely that the antigen to be detected must be common to many tumors of the same origin.

Table (1): Summary of the physicochemical properties of the antigens

Molecular feature	**Method**	**Antigen from**	
		LT-110 (LTAA –2a)	LT –105 (LTAA-2b)
$D_{20,w}$	Sephadex G-200	$5.0_x10^{-7}cm^2sec^{-1}$	$5.15_x10^{-7}cm^2sec^{-1}$
$S_{20,w}$	Sucrose gradient	4.24S	4.24S
Stokes radius	Sephadex G-200	40 A^o	39.4 A^o
M_r	Sephadex G-200	108,000	98,000
M_r	SDS-PAGE	42,000	46,000
M_r	Svedberg equation[a]	77,000	76,000
M_r	Hedrick and Smith	73,000	n.d.
Subunit M_r	Gel chromatography in guanidiner/ DTT	25,000	25,000
f / f_o	Calculated from M_r and ν[b]	1.42	1.40

a-------- $M_r = \frac{RTS}{D(1-\bar{v}p)}$ where Mr is the molecular weight, R is the gas constant, T is the absolute temperature, S is the sedimentation coefficient, D is the diffusion coefficient, $\bar{v}$ is the partial specific volume, and p is the density of the solvent.

b------- $f / f_o = \frac{a}{(\frac{3\bar{v}M_r}{4pN})^{1/3}}$ where f/f_o is the frictional ratio, a is the Stokes radius.

References

1. Lo Gerfo,p. *Lung Cancer, Natural History, Prognosis, and Therapy*., Academic Press, New York, pp 81-93,1986.
2. Veltri R.W., Mengoli, H.F., Maxim, P.E., West fail, S., copo, J.M., Huang, C.W.and Sprinkle, P.M. *cancer* res.Vol. 37. pp.1313-1322, 1977.
3. Frost M.J.,Rogers, G.T.and Bagshawe, K.D.*Br.J. Cancer*,Vol. 31,pp.379-386. 1975.
4. Braats J.A., MeIntire, K.R., Princle G.L., Kortright, K.H. and Herberman, R.B.1978 J.*Natl cancer Inst.*.
5. Al-Atrakchi, S.A.M., "*Protein Engineering of Carcinoembryonic Antigen and Their Receptors Located in Malignant Mammary Tissues*" Ph.D Thesis, College of Science, Baghdad University,2002.
6. Braatz J.A.and McIntire, K.R. *Biochem*., 7, pp. 495-509,1977.
7. Davis B.J. Ann. N.Y. *Acadi Sci*., 121,pp. 404-427.1964.
8. Weber K. and Osburn, M.J. *Biol.Chem*.,Vol. 244, 4406 – 4412,1969.
9. Lowry O.H.,Rosebrough, N.J., Farr, A.J. and Randail, A.J. *J.Biol.Chem*.,Vol.193 ,pp. 265-275. 1951.
10. Martin R.G. ,and Ames, B.N. *J.Biol. Chem*., Vol. 236,pp. 1372 – 1379 ,1961.
11. Andrews P., *Biochem. J.*, Vol. 96,pp. 595 – 506,1965.
12. Hedrick J. T., and Smith, A.*J. Arch. Biochem. Biophys*., Vol. 126,pp.155-64,1968.
13. Siegel,L.M.and Monty, K.*J. Biochim, Biophys. Acta*, Vol.112,pp. 346 – 362,1966.
14. Mori M., Mim ori, K.,Ueo., H., and et . al *Int. J. Cancer* ,pp .68-739,1996.
15. Segrest J.P.,Jackson , R.L., AACKSON, R.L.,Andrews, E.P., and Marchest, V.T. *Biochem.Biophys. Res. Comm.*, Vol. 44, pp.390 – 395,1971.
16. Tanford C., *Physical Chemistry of Macromolecules*, John Wiley , New York, pp. 317 – 456 ,1991.