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Purification of a Cancer-associated Protein Factor in Serum of Lung Cancer Patients*

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Abstract—*The serum factor responsible for the humoral leukocyte adherence inhibition (H-LAI) reaction in lung cancer patients has been purified. It is precipitable by ammonium sulfate between 30–70% saturation. On DEAE ion exchange chromatography activity is eluted in the 0.12–0.2 M acetate fraction. The serum factor has affinity for Con A. This gives evidence for a glycoprotein nature of the factor. Electrophoresis indicates an apparent mol. wt of 71,000 dalton. The data suggest that the protein can be separated into subunits of 43,000 and 28,000 dalton under reducing conditions. Isoelectric focusing gives a mean pI of 4.5. The applied fractionation procedure gave a 1120-fold purification relative to lung cancer serum. Antiserum against the purified factor has been produced in rabbits. The factor can be demonstrated in high concentrations in serum of lung cancer patients, but is also found in smaller quantities in sera of other types of cancer. Minor quantities are present in normal serum. The results suggest that quantitation of the H-LAI factor can be used in cancer diagnosis.*

INTRODUCTION

IMMUNODIAGNOSIS of cancer has so far been centered around assay systems based on detection of tumor cell products, e.g. increased secretion of hormones and enzymes, oncofetal antigens and other tumor-related antigens [1–5]. Determinations of such products appear to be of great prognostic and monitoring importance, but will probably not be suitable for early diagnosis. Their limitation is due to the fact that detectable levels will only be obtained when the tumor mass has grown to a certain size.

Recently, a test has been developed in our laboratory which appears to give an expression of humoral antitumor reactivity [6–10]. In this assay serum from the person under study is mixed with the relevant antigen and the reaction is measured by the use of trypsinized, normal leukocytes as indicator cells [6]. The test is called the humoral leukocyte adherence inhibition (H-LAI) assay as the technical procedure is based on that of the original hemocytometer leukocyte adherence inhibition assay [11].

Previous studies have demonstrated the possible potential of the H-LAI assay as an immunodiagnostic test in cancer [6–10]. It has been shown that more than 90% of lung cancer patients [7] and about 70–80% of breast cancer patients in stages I and II will respond in the assay in a specific manner [6]. The response frequency in persons with high risks for developing cancer is found to correlate to observed preneoplastic changes [9].

The serum factor responsible for the observed H-LAI reaction forms stable complexes with the tumor-associated antigen [2], and the serum factor can be specifically absorbed on antigen-coupled columns [12]. This antitumor immune factor can be demonstrated in the preclinical period of lung cancer. Positive reaction has been found nearly 5 yr prior to the clinical verification of the disease [8].

In this communication the purification and characterization of the serum factor responsible for the H-LAI reactivity in lung cancer patients is reported. This factor may prove useful in early cancer diagnosis.

MATERIALS AND METHODS

Steps in the H-LAI factor purification

All procedures were carried out at 4°C unless otherwise stated. H-LAI analyses have been performed to define specific activity in each subfraction in the purification.

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Collection of serum (fraction I). Serum from lung cancer patients was obtained from blood samples drawn one day after admittance to the Norwegian Radium Hospital. Pooled serum from several lung cancer patients was used in the purification procedure.

Precipitation by ammonium sulfate (fraction II). Pooled lung cancer serum (350 ml) was centrifuged at 20,000 *g* for 15 min and administered to sequential precipitation by adding saturated ammonium sulfate in phosphate-buffered saline (PBS, pH 7.0). After stirring for 30 min the suspension was centrifuged at 20,000 *g* for 15 min. The precipitate between 30 and 70% saturation was dissolved in PBS and dialyzed extensively.

Ion exchange chromatography on a DEAE-cellulose (diethylaminoethyl cellulose) column (fraction III). Dissolved precipitate was fractionated on a DE-52 column (Whatman Uniscience Ltd., Cambridge) (5 × 40 cm) equilibrated with ammonium acetate buffer (0.01 M, pH 6.3). A linear gradient of ammonium acetate (0.01–0.3 M) was achieved by use of an Ultragrade gradient mixer (LKB, Bromma, Sweden). A total volume of 4200 ml was used, with an approximate flow rate of 80 ml/hr. Ten-milliliter fractions were collected and the specific conductivity was measured in each fraction.

Gel filtration on Sephadex G-200 (fraction IV). A Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (1.6 × 98 cm) was eluted with phosphate-buffered saline (PBS, 0.5 M NaCl, pH 7.2) at a flow rate of 12 ml/hr. Void volume and molecular weight distribution was determined using Dextrane Blue 2000, bovine serum Albumin (BSA) and ovalbumin (Sigma Chemical Co., St. Louis, MO, U.S.A.).

Concanavaline A (ConA)-affinity chromatography (fraction V). Sodium acetate solution (0.03 M, 1 M NaCl, 10^{-3} M $CaCl_2$, $MgCl_2$ and $MnCl_2$, pH 6.0) was used for equilibration of a ConA-Sepharose (Pharmacia Fine Chemicals) column (2.6 × 30 cm). The flow rate was 20 ml/hr and 10-ml fractions were collected. For desorption the same buffer containing α-methyl-D-mannoside (0.3 M) was used.

Affinity chromatography on antibody-coupled columns (fractions VI and VII). Anti-albumin and anti-human serum proteins (Dakopatts, Copenhagen, Denmark) were coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) according to the instructions given by the manufacturer. The columns (2.2 × 12 cm) were eluted with a bicarbonate buffer (0.1 M, 0.5 M NaCl, pH 8.0). As desorption agent, the same buffer with guanidine–HCl (4 M) was used. A flow rate of 20 ml/hr was applied.

Polyacrylamide gel electrophoresis (PAGE) (fraction VIII). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed, using a modified Laemmli discontinuous buffer system [13]. Stacking gel was 4% PAA, and separating gel was 7.5–10% polyacrylamide (PAA). To avoid reducing conditions, mercaptoethanol and heating of the samples were omitted.

For preparative use, PAGE was performed without SDS and mercaptoethanol. The gels were sliced and eluted with an appropriate buffer (see below). High- and low-molecular-weight standards were purchased from Pharmacia Fine Chemicals.

Isoelectric focusing (IEF) (fraction IX). Isoelectric focusing was performed in a preparative and analytical manner, and focusing was carried out in both columns and gel rods.

In columns. Electric focusing was performed according to the LKB manual. A LKB 8101 (LKB) column (110 ml capacity) was used. The electrolysis was carried out for 48 hr at 6°C with constant power not exceeding 3 W. A 1% (v/v) ampholine (LKB) covering the pH range 3.5–7.0 was used. The pH in each fraction was measured at 6°C.

In gel rods. The procedure of Wrigley [14] was used. Polyacrylamide (7.3%) (BioRad Lab, Richmond, CA, U.S.A) and ampholines (1% v/v) (LKB) in the pH range 3.5–7.0 were used. The electrolysis was performed for 18 hr at 6°C with constant voltage (200 V). The gels were cut in a gel slicer (BioRad Lab). For pH determination, the slices were eluted in degassed 0.01 M KCl.

Protein elution from PAA-gels

Gel fractions from several gels were pooled and eluted for 2 days with at least four buffer changes (PBS). Traces of radiolabelled protein had been added before electrophoresis so that efficiency in elution could be followed.

Concentration of samples

Concentration of fractions were carried out in ultra filtration cells (Amicon, Lexington, MA, U.S.A.) with PM 10 filters (Diaflow membranes, Amicon, Lexington, MA, U.S.A.) under N_2 pressure. For smaller volumes Satorius cells with collodium bags (Satorius, Goettingen, F.R.G.) were used.

Protein assay

Protein concentration of the column effluents were estimated spectrophotometrically by absorption at 280 nm. In samples small in volume and low in protein, the protein concentration was estimated in polyacrylamide electrophoresis by comparison with known amounts of protein

standards, BSA and trypsin inhibitor (Sigma). All stainings were done with Coomassie Blue R-250 (BioRad Lab).

Antiserum

Antibodies against the purified H-LAI factor were obtained by immunizing rabbits subcutaneously at multiple sites with a 1:3 mixture of the factor and Freuds complete adjuvant. The titer was analyzed every 14 days and if a decreasing titer was found, a booster injection was given.

Radiolabelling of proteins

Labelling of the proteins was performed by the Iodogen technique [15]. Free ^{125}I was separated on a P-150 (BioRad Lab) column, eluted with phosphate buffer (0.1 M, 0.1% BSA, pH 7.0).

Crossed immunoelectrophoresis

The technique described by Clarke and Freeman [16] was used. One-percent agarose (Litex HSA, Denmark) in Tris–barbitone buffer ($I = 0.5$, pH 8.6) was used. In some instances intermediate gels were used. The first-dimension electrophoresis was performed at 10 V/cm for about 1.5 hr, while second-dimension electrophoresis was carried out at low field conditions with 1.5 V/cm for 18 hr. Antisera against serum proteins were purchased from Dakopatts.

Rocket immunoelectrophoresis

This electrophoresis was performed according to the standard procedure of Laurell [17]. Low field conditions with 1.5 V/cm for 18–20 hr were used. Agarose solutions and buffers corresponded to that used for crossed electrophoresis.

Double immunodiffusion

The analysis was carried out in the agarose solution mentioned above. The thickness of the agar was 1.5 mm, with well diameters of 3 mm. A sample of 10 μl per well and appropriate concentrations of the antisera were allowed to diffuse for at least 72 hr at 20°C.

H-LAI analyses

Indicator cells. Blood from normal, healthy blood donors was purchased from Røde Kors Blodsenter, Oslo, Norway. The mononuclear cells were separated according to the method of Bøyum [18]. The cells were washed and treated with 0.025% trypsin, as described elsewhere [6, 7]. If not used immediately, the trypsin-treated indicator cells were cryopreserved and thawed upon need according to the procedures described previously [9].

Cancer antigens. Potassium chloride (3.5 M) extracts from the cell lines Calu-1 and MCF-7 were used as the lung and breast cancer antigen respectively. The Calu-1 cell line has been isolated from a squamous cell carcinoma of the lung by Dr J. Fogh, Sloane Kettering Institute, New York. The MCF-7 cell line was derived from a pleural effusion of a breast carcinoma and kindly provided by Dr M. Rich, AMC Cancer Research Center, Lakewood, CO.

H-LAI technique. Details of the procedure have been published elsewhere [7]. In brief, serum (0.5 μl) and antigen (5 μg protein) were incubated in a total volume of 150 μl of Minimum Essential Medium (MEM) at 4°C for 1 hr. Cryopreserved trypsin-treated indicator cells (10^6) were added to the above mixture and incubated for 30 min at 37°C. Aliquots of the cell suspension were subsequently transferred to hemocytometers and incubated for another hour at 37°C. At the end of the incubation, the cells were counted in nine predetermined squares on each side of the hemocytometer. Between 4000 and 5000 cells were counted by the use of an Image Analyzer. The cover glass was removed, the surface gently rinsed to remove non-adherent cells and the same squares were recounted. Each test was performed in duplicate. The response of the test is expressed by the LAI index:

$$\frac{A_a - A_p}{A_a} \times 100,$$

where A_a and A_p represent the percentage of adherent cells in the absence and presence of antigen respectively. On the basis of previous experience, an LAI index greater than 10 was taken as a positive test [7, 8].

Calculation of H-LAI units. Under the defined assay conditions, previous findings have revealed the need for about 0.5 μl serum from a lung cancer patient to give a positive LAI value. This amount of serum is thus defined as one H-LAI unit. This gives an average of 2000 H-LAI units per ml of lung cancer serum. The specific activity is calculated as the number of units per mg protein in the fraction.

RESULTS

Purification of the H-LAI factor

Table 1 gives a summary of the steps in the purification of the factor responsible for the H-LAI reactivity in serum from lung cancer patients.

The H-LAI factor was precipitated in 30–70% saturated ammonium sulfate solution [12]. The dissolved precipitate was separated on an ion exchange DE-52 column by a linear ammonium acetate gradient (pH 6.3). The active fraction was eluted by 0.12–0.20 M ammonium acetate

Table 1. Purification of the H-LAI factor from sera of lung cancer patients

	Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
I	Pooled serum	28,000	7.0×10^5*	25	1	100
II	$(NH_4)_2SO_4$ precipitate	1,688	9.0×10^4	53	2.1	12.9
III	DEAE cellulose (DE-52)	320	2.5×10^4	78	3.1	3.6
IV	Sephadex G-200	54	1.5×10^4	274	11	2.1
V	ConA-Sepharose	12.8	8.6×10^3	627	26.9	1.2
VI	Anti-albumin Sepharose	9.5	6.9×10^3	724	29	1.0
VII	Anti-total-h serum protein Sepharose	3.2	4.0×10^3	1250	50	0.6
VIII	PAGE-sliced	0.3	2.0×10^3	4608	184	0.3
IX	IEF	0.06†	1.7×10^3	28,000	1120	0.24

*The H-LAI activity of the pooled cancer pulm serum was 2000 U/ml.
†Protein concentration was estimated in PAGE by comparison of the protein content in the samples with known amount of protein standard.

(specific conductivity, 9.5–15.5 mS). This fraction was concentrated and further fractionated on a Sephadex G-200 column. Figure 1 shows the elution profile and the corresponding H-LAI activities. The H-LAI-reactive protein was found to eluate with a distribution coefficient corresponding to molecular weight of 30–80 kD.

Further purification was done on a ConA-affinity column. The factor had affinity to ConA but could be desorbed with 0.3 M α-methyl-D-mannoside. This gives strong evidence for a glycoprotein nature of the factor.

As the factor was found to co-eluate with BSA on the Sephadex G-200 column, attempts were made to get rid of the corresponding human serum albumin and other common serum proteins. Consequently the active H-LAI fraction was subjected to both anti-human-albumin and anti-human-serum protein Sepharose-4B-affinity columns. The 'flow-through' fractions from these columns retained the H-LAI activity. Due to the limited amounts of protein left at this stage, further column separations were avoided. The active fractions were subsequently run on a polyacrylamide gel electrophoresis and the gel rods sliced.

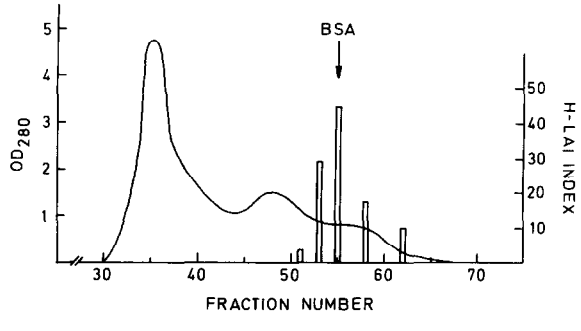

Fig. 1. Elution profile of the active fraction from the ion exchange column on Sephadex G-200. The open bars represent the H-LAI activity measured in the corresponding fraction number.

The H-LAI activity was eluted in the fraction with 0.40–0.57 in relative migration distance. As a final step, the active fraction was subjected to separation on isoelectric focusing in polyacrylamide gel rods. The gel rods were sliced and monitored for reactivity and the active fraction was pooled, eluted and concentrated. This fraction was used for immunization.

It should be emphasized that the number of H-LAI units and the specific activity given for each fraction in the purification procedure are estimates. Complete dose–response curves in each separation step were beyond the capacity of the H-LAI method. With this reservation, the fractionation procedure gives a 1120-fold purification of the H-LAI factor relative to the lung cancer serum.

Characterization

Molecular weight determination. A small proportion of the purified fraction for immunization was labelled with ^{125}I and run on SDS-PAGE. Figure 2 shows the radioactive profile under non-reducing and reducing conditions. The protein found at 71 kD was identified as the protein factor responsible for the H-LAI reaction. Under reducing conditions a peak appeared at 43 kD and the relative size of the 28-kD peak increased, suggesting that the 71-kD glycoprotein can be split into two subunits.

Isoelectric point (pI). Estimation of pH in the sliced gel-rods is subject to uncertainties. In order to assure a more reliable value of the actual pI of the factor, these measurements were done on a more crude preparation with electrofocusing in a column. Figure 3 shows the results. The reactivity was found in the pH range 4.14–4.85, with a mean pI of the factor estimated to pH 4.5.

Analyses of the H-LAI factor against antisera of known serum proteins. The purified H-LAI factor fraction was analyzed on both crossed

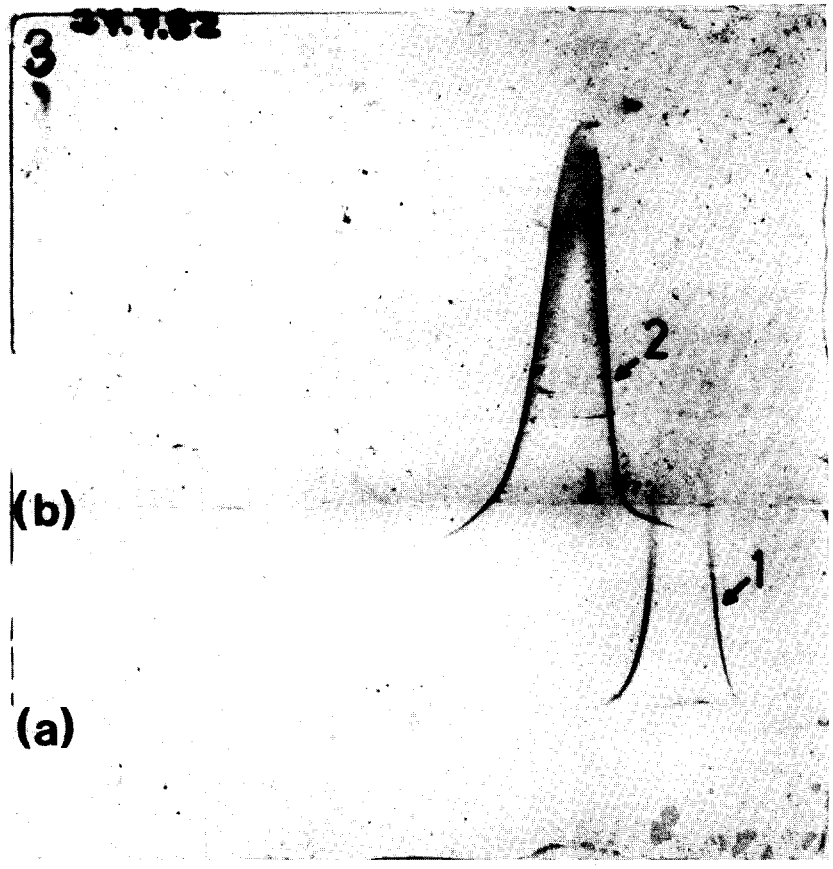

Fig. 4. Crossed immunoelectrophoresis of the H-LAI factor. 1.dim: 10 μl *of the immunosate. 2.dim:* (a) *intermediate gel with anti-albumin;* (b) *reference gel with antibodies raised against the immunosate. Precipitate No.*1: *albumin. Precipitate No.*2: *the H-LAI factor.*

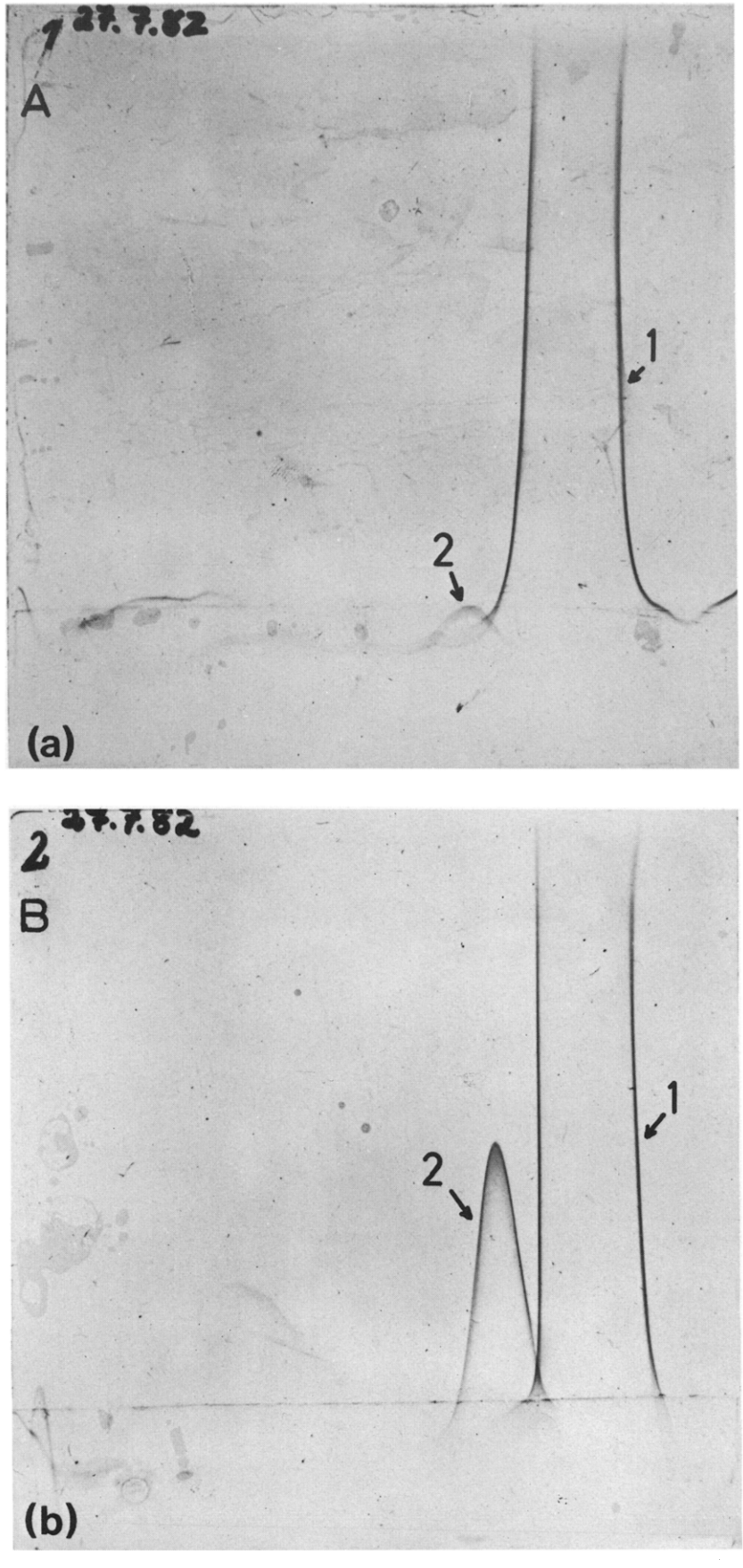

Fig. 5. Crossed immunoelectrophoresis with anti-H-LAI antiserum on normal and lung cancer serum. 1.dim: 10 µl of normal serum (A) and lung cancer serum (B) diluted 1:20. 2 dim: reference gel with antiserum raised against purified H-LAI factor.

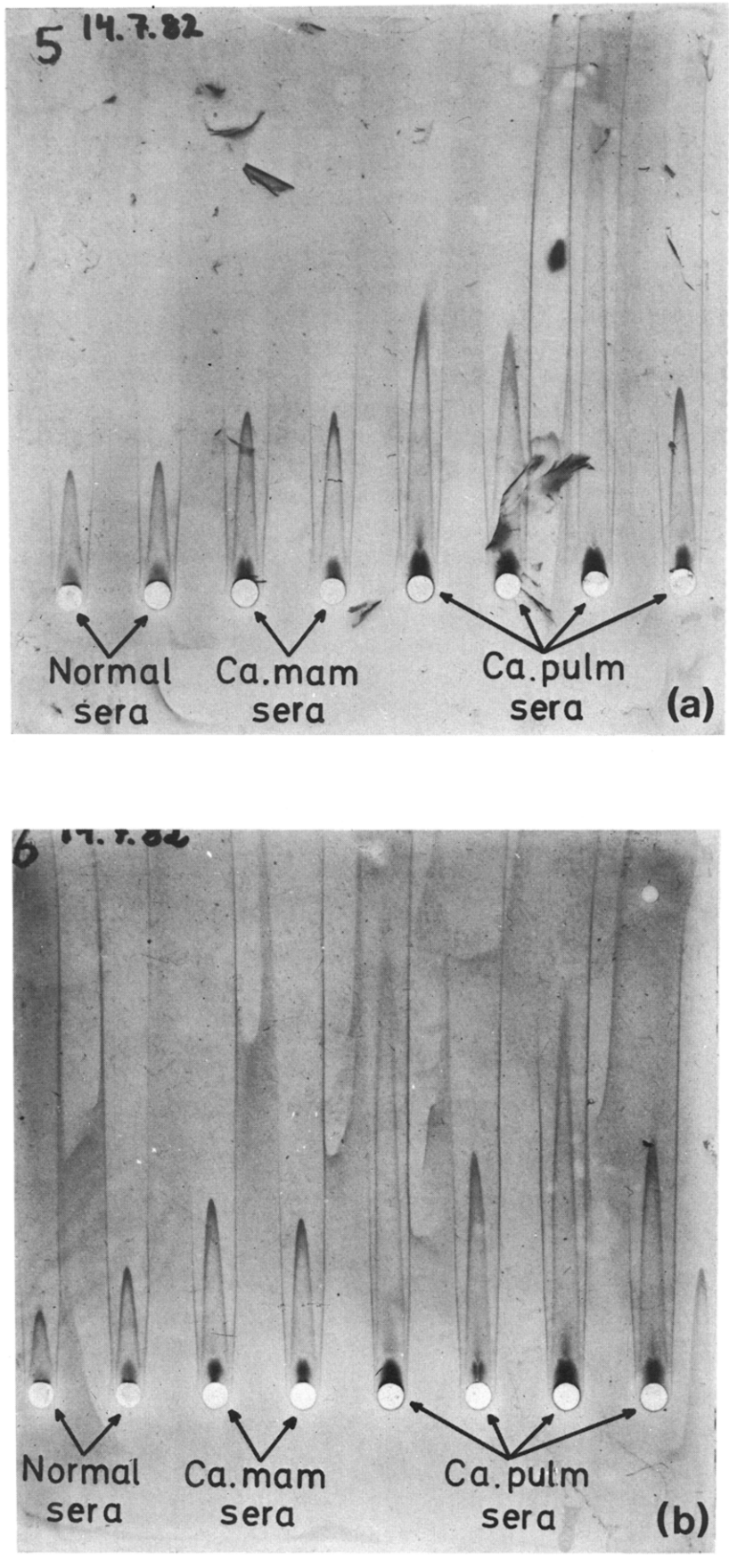

Fig. 6. Rocket immunoelectrophoresis against the anti-H-LAI-factor antiserum of controls and cancer patients. 10 µl/well of normal-, breast and lung cancer serum diluted 1:20. 500 µl of anti-H-LAI-factor antiserum was used per plate.

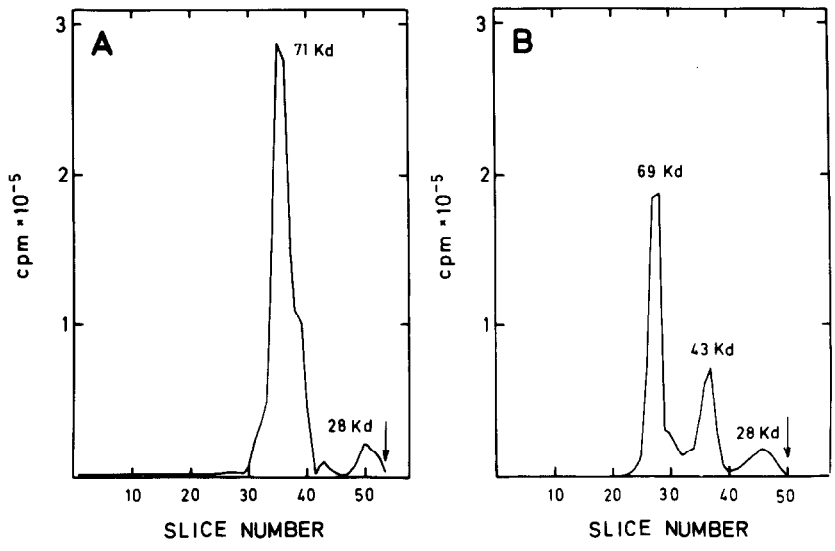

Fig. 2. SDS-PAGE on the purified H-LAI factor. Purified ^{125}I-labelled H-LAI factor was run on a 7.5% PAA gel rod. The gel was sliced and each slice counted for radioactivity. (A) *Non-reducing conditions.* (B) *Reducing conditions.*

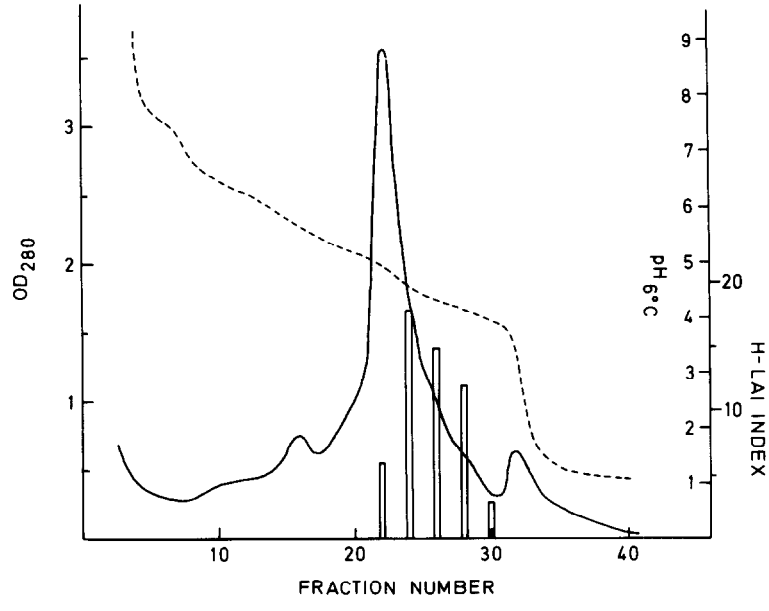

Fig. 3. The ConA-absorbed fraction on IEF. The open bars represent the H-LAI activity measured in the corresponding fraction number. Activity was found in the area of 4.15 <pH <4.85, with a mean pI for the H-LAI factor of 4.5.

immunoelectrophoresis and double immunodiffusion against antisera directed against several serum proteins. No identity was found with α-fetoprotein, β_2-microglobulin, transferrin, C3-complement, IgA, IgG, IgM, albumin, pregnancy-specific protein (SP-1) and α_1-antitrypsin. However, it could be seen that the purified fraction was contaminated with small amounts of albumin, despite the two immunosorbent steps.

Specificity of the antiserum. Figure 4 shows the crossed immunoelectrophoretic pattern obtained between antiserum and the immunosate. The intermediate anti-albumin gel verifies impurities of albumin. Except for the albumin, the obtained antiserum seems to recognize only one major protein in the immunosate.

In order to confirm specificity against the factor responsible for the observed H-LAI reaction, the produced antiserum was used for blocking experiments in the H-LAI assay. As the antiserum contains some specificities against albumin, direct blocking was not successful due to the influence of immune complexes formed with this protein. To avoid this problem, serum was absorbed on the anti-H-LAI-factor antibody column and reactivity was measured before passage through the column as well as on the desorbed fraction. The results are given in Table

2. As can be seen from the results of the desorbed fraction, the antiserum is picking up the proteins that are giving the specific H-LAI reaction. This implies that the antiserum produced has specificities for the H-LAI factor

Anti-H-LAI-factor antiserum in lung cancer diagnosis. The anti-H-LAI-factor antiserum was studied in crossed immunoelectrophoresis for the possible use in lung cancer diagnosis. The panels in Fig. 5 show the crossed immunoelectrophoretic pattern given on normal (A) and lung cancer serum (B). Precipitate 1 is identified as albumin, while precipitate 2 is confirmed as the H-LAI factor.

In a limited study the antibody against the factor has been used on a small group of cancer patients. The concentration of the H-LAI factor in these sera was determined by rocket immunoelectrophoresis (Fig. 6). The concentrations were found to be higher in sera of lung cancer patients than is sera of breast cancer patients. Comparatively lower concentrations were found in sera of normal individuals.

DISCUSSION

The purification and characterization of the humoral factor responsible for the H-LAI activity in lung cancer patients are reported. The H-LAI factor was found to be a glycoprotein of 71,000 dalton. The data suggest that under reducing conditions it can be split into 28,000- and 43,000-dalton subunits. The IEF gave a mean pI of 4.5.

It was of interest to establish whether the H-LAI factor represents a known or a new type of serum proteins. Double immunodiffusion and crossed immunoelectrophoresis against a battery of antisera showed no identity to α-fetoprotein, β_2-microglobulin, transferrin, C3-complement, IgA, IgM, IgG, albumin or pregnancy-specific protein (SP-1). From the elution profiles and characteristics given for the H-LAI factor, it is clearly not an IgG molecule nor is it likely to be any fragments of IgG. The elution on the anti-human-serum protein column should give an indication for the H-LAI factor as a protein not found in high concentrations in normal serum.

A recent report [19] focused on the *in vitro*, immunosuppressive effect of the increased concentration of certain acute-phase reactant proteins in cancer. Except for α_1-antitrypsin (54 kD) the molecular weight of any of these proteins is not within the range found for the H-LAI factor. Separate studies have revealed no identity with α_1-antitrypsin.

Many of the characteristics of the H-LAI factor are similar to those reported for soluble mediator substances, such as the migration inhibitory factor (MIF) [20], interferon-γ (IFN-γ) [21], most antigen-induced lymphokines, and suppressor (SF) and helper (HF) factors [22]. Both MIF and other antigen/mitogen-induced lymphokines are found to be precipitable by ammonium sulfate between 50 and 80% saturation [23]. Optimal precipitation of the H-LAI factor has been found to be between 50 and 85% saturation (data not shown). Further, these mediators elute on DEAE ion-exchange chromatography at a relatively high salt concentration. Most of these factors are also found to be glycoproteins with affinity for ConA. This coincides with the observations made with the H-LAI factor.

MIF is one of the lymphokines most extensively studied, and two major molecular weights of 67 and 12 kD exhibit the activity. There are reports that suggest MIF to be antigen specific [20]. Although first thought to be 40–70 kD, the basic structure of IFN-γ consists of 20- and 25-kD molecular forms [24]. In mouse systems it has been found that IFN-γ can be antigen-specific and MHC-restricted [25]. However, when analyzed using isoelectric focusing, activity is most frequently found in the range of pH 5.7–7.0 [26]. Antigen-specific helper and supressor factors are found in the range 35–70 kD [27, 30]. Binz and Wigzell [31] have isolated a T cell receptor in the rat, containing a single polypeptide chain of 70 kD.

Sera from healthy persons seldom give positive reactions in the H-LAI assay. If this new characterized H-LAI factor should have any significance to what is measured in the H-LAI assay, it would be expected that the concentration

Table 2. Specific absorption of the H-LAI factor on an anti-H-LAI-factor coupled column

	Before absorption		Desorbed from the anti-H-LAI factor column*	
	MCF-7	Calu-1	MCF-7	Calu-1
Lung cancer serum	4	20	-1	12
Breast cancer serum	17	10	2	2

*Antiserum against the H-LAI factor was coupled to Sepharose 4B. Lung- and breast cancer serum was absorbed onto the column and eluted with guanidin-HCl (4 M) as desorbant. The desorbed fractions were dialyzed and tested for specific H-LAI activity.

of the factor should be enhanced in lung cancer patients. The anti-H-LAI-factor antiserum was therefore evaluated for possible quantitation of the H-LAI factor in a small group of cancer patients.

In this study (Fig. 6) the concentration of the H-LAI factor in lung cancer patients was found to be higher than in breast cancer patients. Whether this reflects direct quantitative differences or whether the antiserum possesses specificities against both common and unique determinants on the factor remains to be seen. One explanation could be that the LAI factor contains a common antigenic site shared by all LAI factors and a tumor antigen recognition site that confers the specificity in LAI. The possibility also exists that one type of H-LAI factor could be directed against unique determinants of the particular cancer type in question, while others could be directed on the regulation of common cancer-related antigens. Work is underway to purify the corresponding H-LAI factor from breast cancer serum. The fact that small amounts of the factor seems to be present in normal serum supports the assumption of the factor as being an antigen-specific glycoprotein with a more general and conserved role in the immune regulation, most probably residing within the normal family of lymphokines.

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