J. Biosci., Vol. 16, Number 6, December 1991, pp 223–233. © Printed in India.

Plasminogen activator: Isolation and purification from lymphosarcoma of ascites bearing mice

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MS received 14 January 1991; revised 12 August 1991

Abstract. Plasminogen activator secreted by lymphosarcoma (ascites) of mice was purified up to 163-fold by ammonium sulphate fractionation at 35% saturation and chromatography on *p*-aminobenzamidine-Sepharose 4B. The purified activator contained specific activity of 9980 IU/mg. The plasminogen activator displayed homogeneity by polyacrylamide slab gel electrophoresis and high performance liquid chromatography. The activator consisted of a single polypeptide chain with an apparent molecular weight of 66,000 daltons as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis under reducing conditions as well as gel filtration on Sephadex G-100. Distinct differences between this activator and urokinase were discernible in respect of specific activities, fibrin affinity and immunochemical properties. The lymphosarcoma activator appears to be of tissue-type origin since it showed gross similarity to standard tissue plasminogen activator in terms of modes of binding to fibrin and immunological attributes.

Keywords. Plasminogen activator; urokinase; fibrinolysis; Fibrin; lymphosarcoma.

1. Introduction

Plasminogen activators form a key component of fibrinolytic system with a high specificity for plasminogen yielding the active enzyme plasmin through the hydrolysis of the Arg_{560}—Val_{561} peptide bond (Ranby and Brandstrom 1988; Collen and Gold 1990). Activation also serves as an important source of localized proteolytic activity during tumour invasion, ovulation, cell migration and various physiological processes (Dano *et al* 1985; Loskutoff 1988; Vansetten *et al* 1989). Two functionally and immunologically distinct enzyme species of plasminogen activators (PA) have been identified, the urokinase-type and tissue-type activators present in a wide variety of tissues, body fluids, malignant tumours and transformed cell lines (Hekman and Loskutoff 1987; Gross *et al* 1988). These enzymes have been purified and shown to be serine proteases with high specificity to plasminogen (Prager *et al* 1986; Hajjar and Hamel 1990). Reports of Saksela (1985), Cajot *et al* (1986) and Gross *et al* (1988) suggest that rapidly dividing cells such as tumour cells contain high amounts of plasminogen activator activities. While working with rat Yoshida sarcoma (Nulkar *et al* 1983) and lymphosarcoma system of mice it was observed that fibrinolytic system played an important role during propagation of tumour and metastases. It was therefore of interest to examine lymphosarcoma as a source of plasminogen activator. The present paper relates to the purification and characterization of plasminogen activator secreted by lymphosarcoma of mice.

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2. Materials and methods

2.1 *Materials*

CNBr-activated Sepharose 4B, lysine Sepharose and Sephadex G-100 were obtained from Pharmacia Fine Chemicals AB (Sweden). Human urokinase was purchased from Leo Pharmaceutical Laboratories (Copenhagen) while standard tissue-plasminogen activator (≃500,000 IU/mg fibrinolytic activity) bovine serum albumin, ε-aminocaproic acid and the electrophoresis calibration kit were the products of Sigma Chemical Co., USA. D-Val-Leu-lysine *p*-nitroanilide, bovine fibrinogen (S-2251) were obtained from Kabi, Sweden. Rat plasminogen was prepared by affinity chromatography of plasma on lysine-Sepharose 4B (Deutsch and Mertz 1970). All the other chemicals used were of Analar grade obtained from the Standard sources.

2.2 *Experimental procedures*

2.2a *Isolation of tumour cells:* Inbred male Swiss mice (8–10 weeks old) originally obtained from the Jackson Laboratories, Bar Harbour, Maine, were fed on stock laboratory diet and maintained in the laboratory. From a spontaneously developed lymphosarcoma grown in this laboratory (Podval *et al* 1984) an ascitic variant was established as a subline by repeated transfer of tumour cells from peritoneal fluid into the peritoneum of syngeneic Swiss mice (Thakur *et al* 1990). Ascitic lymphosarcoma cells were harvested from mice on the twentyfirst day after initial ip transplantation ($\simeq 10^3$ cells). The tumour cells were harvested in the presence of 10·0 KIU/ml Aprotinin and washed with buffer containing 0·05 M Tris-HCl, pH 7·5, 0·13 M NaCl, 0·025 M KCl, 0·0025 M $MgCl_2$ by centrifugation at 600 *g* for 5 min. The washing procedure was repeated twice for removal of traces of blood cells.

2.2b *Purification procedure*: The washed cells were suspended in 4 vol of 0·25 M sucrose, 0·05 M Tris-HCl, pH 7·5, 0·1% Triton X-100, homogenized and centrifuged at 20,000 *g* for 30 min. The clear supernatant fraction was dialyzed against 0·05 M Tris-HCl buffer pH 7·5 overnight at 4°C and precipitated with ammonium sulphate at 35% saturation. The precipitate was suspended in 0·3 M Tris-HCl containing 0·01% Triton X-100 and was sedimented at 25,000 g for 30 min. The supernatant was dialyzed against 0·05 M Tris-HCl pH 8. The most frequently used affinity matrix for purification (Gilbert and Wachsman 1982) procedure is *p*-amino-benzamidine-Sepharose Chromatography which was utilized for the purification of plasminogen activator of lymphosarcoma. The dialyzed supernatant was passed directly onto a column (0·8×10cm) of amino-caproyl-*p*-aminobenzamidine-Sepharose 4B that had been equilibrated previously in 0·05 M Tris-HCl buffer, pH 7·5 followed by 50 ml washings of the same buffer. The column was then washed sequentially with 50 ml each with (i) 0·05 M Tris-HCl, 0·8 M NaCl, pH 7·5, (ii) 0·05 M Tris-HCl, 1·0 M NaCl, pH 8 and (iii) 0·05 M Tris-HCl, 0·5 M NaCl, pH 8 with a flow rate of 8 ml/h. The washings of the column were collected in 10 ml fractions and checked for protein [E_{280}] and enzyme activity expressed as plasmin units. The enzyme was eluted (1 ml fraction/tube) from the column in 25 ml using 0·2 M arginine contained in 0·05 M Tris-HCl buffer with 0·5 M NaCl and 0·01% Triton A-100, pH 8.

2.2c *Biological activity*: (i) Caseinolytic activity of plasmin was assayed using 20 mg of casein with 2 Caseinolytic units contained in 0·1 ml plasminogen along with the activator, or urokinase (200 units) in a total volume of 2 ml at 37°C for 30 min. The reaction was arrested by the addition of 2 ml of 1·7 M perchloric acid and the supernatant was read at 280 nm by the procedure of Johnson *et al* (1969). (ii) The fibrinolytic activity of plasminogen activator was determined by the clot lysis time method as described by Electricwala *et al* (1985) comparing with standard urokinase preparation. The reaction mixture contained 500 μg of purified fibrinogen, 100 μg of plasminogen, 0·2 ml of activator or urokinase (10 IU), 0·05 ml of thrombin (40 NIH units) and 0·5 ml of 0·02 M sodium phosphate buffer, pH 7·4, 0·15 M NaCl and 0·01% Tween 80. After incubation at 37°C the time of complete lysis of the clot formed was recorded, (iii) Amidolytic activity of the activator was measured according to the method of Cheung *et al* (1986) in a total volume of 1·0ml. 0·1 ml of S-2251 (0·3 mM final concentration) was added to 0·01 ml activator or urokinase (10 IU) followed by 0·2 ml plasminogen (3 mM) and 0·6 ml of 20 mM phosphate buffer containing 0·1 M NaCl, pH 7·4. The release of *p*-nitroaniline was monitored at 405 nm at 37°C using Gilford spectrophotometer. The activity has been expressed in urokinase equivalent IU/μg by comparison with the International Reference Preparation for urokinase. The activity of urokinase was checked prior to each use. Simultaneously, the activity was compared with standard commercial preparation of t-PA for identifying the nature of activator.

2.2d *Molecular weight determination*: The molecular weight of plasminogen activator was determined by gel filtration on Sephadex G-100. The Sephadex column (1·5 × 60 cm) equilibrated with Tris-HCl buffer, pH 7·5 was calibrated in the presence of Standard proteins including cytochrome C (12,500), trypsin (24,000), ovalbumin (45,000) and serum albumin (66,000).

High performance liquid chromatography (HPLC) of PA or Standard protein markers was performed on a 0·75 × 60 cm prepacked TSK G 3000 SW column (Pharmacia, LKB, Germany) using 50 mM Tris-HCl, 100 mM NaCl, pH 7·2 at 280 nm.

2.2e *Other analytical procedures*: Protein content was determined by the method of Lowry *et al* (1951). Polyacrylamide gel electrophoresis (PAGE) was carried out in 7·5% polyacrylamide slab gels (Lugtenberg *et al* 1975). Electrophoresis on sodium dodecyl sulphate (SDS)-polyacrylamide gel slabs was conducted by the method of Laemmli (1970) using 4–15% polyacrylamide gradient gel, under reducing conditions in the presence of 0·1 % 2-mercaptoethanol along with the calibration kit consisting of the standard protein markers of high molecular weights. The gels were stained with Coomassie blue R-250 and destained with 10% glacial acetic acid. In some experiments they were restained with ammoniacal silver solution as described by Wray *et al* (1981).

2.2f *Binding of activator to fibrin*: Bovine plasminogen-free fibrinogen (500μg) isolated by lysine-bound Sepharose 4B column was mixed with 200 μg of activator or urokinase, 0·05 NIH units of thrombin and 0·8 ml of 20 mM sodium phosphate buffer 0·1 M NaCl, pH 7·4. At the end of 5 min the clot formed was incubated at 37°C for 1 h followed by sedimentation at 30,000 g for 10 min. The activity was

assayed in the supernatant by incubating 0·8 ml of the supernatant with 50 μg of plasminogen and 0·3 mM S-2251 at 37°C for 30 min. The absorbance was read at 405 nm after termination of the reaction with 0·1 ml glacial acetic acid as described by Cheung *et al* (1986).

2.2g *Immunochemical method* : Antibodies against lymphosarcoma activator were raised in female rabbits by three fortnightly subcutaneous injections of purified activator (200 μg) in Freund's adjuvant. Antiserum was prepared from the blood drawn from the ear vein, one week after the last injection and was stored at – 10°C. Double immunodiffusion analysis was carried out in 1·5% agar gel as described by Ouchterlony and Nilsson (1973). Wells containing samples were allowed to diffuse overnight by placing the plates at 4°C in a humid atmosphere. The precipitin arcs were stained by 0·25% Coomassie blue and photographed.

3. Results

3.1 *Purification of plasminogen activator of lymphosarcoma of ascites cells*

Preliminary work with lymphosarcoma system indicated that 25% of the homogenate contained substantial amount of PA activity as judged by caseinolytic assay procedure and by the clot lysis time method. For purification of the activator from this source, clear supernatant (20,000 g) fraction was prepared, dialyzed and precipitated by ammonium sulphate at 35% saturation. After extensive dialysis, the supernatant was chromatographed on amino-caproyl *p*-aminobenzamidine Sepharose 4B. The flow-through of the column contained about 3–4% of PA activity. Washings with buffers containing varying concentrations of sodium chloride essentially served to eliminate some of the contaminants associated with the activator. Figure 1 shows the activity bound to the column following elution by 0·05 M Tris-HCl buffer containing 0·5 M L-arginine, 0·01% Triton X-100 and 0·5 M NaCl at pH 8. Fractions 15–40 were pooled to study enzymatic activity. About 0·3–0·4% of the protein applied to the column was present in the peak of activity showing 163-fold enhancement in the specific activity (9980 units/mg). As much as 28% of the activity could be recovered from the eluate (table 1). The final yield of the purified enzyme varied between 0·17–0·18 mg/108 ml of tumour cells harvested from 12 mice.

The biological activities of the activator with reference to caseinolytic, fibrinolytic and amidolytic activities are summarized in table 2. In the presence of activator the time required for plasma clot lysis was comparable with that of urokinase while the standard t-PA preparation displayed marginal difference. The activator showed as much as 65 and 85% caseinolytic and amidolytic activities respectively when compared with urokinase. The binding of activator or t-PA to fibrin clot showed that 59 and 66% of activity respectively were accessible to binding with fibrin while with urokinase there was a lack of affinity to fibrin. This would suggest that lymphosarcoma activator had relatively high affinity to fibrin when compared to urokinase.

Polyacrylamide slab gel electrophoretic analysis of protein samples from each step of purification is shown in figure 2. Although the resolution of protein bands

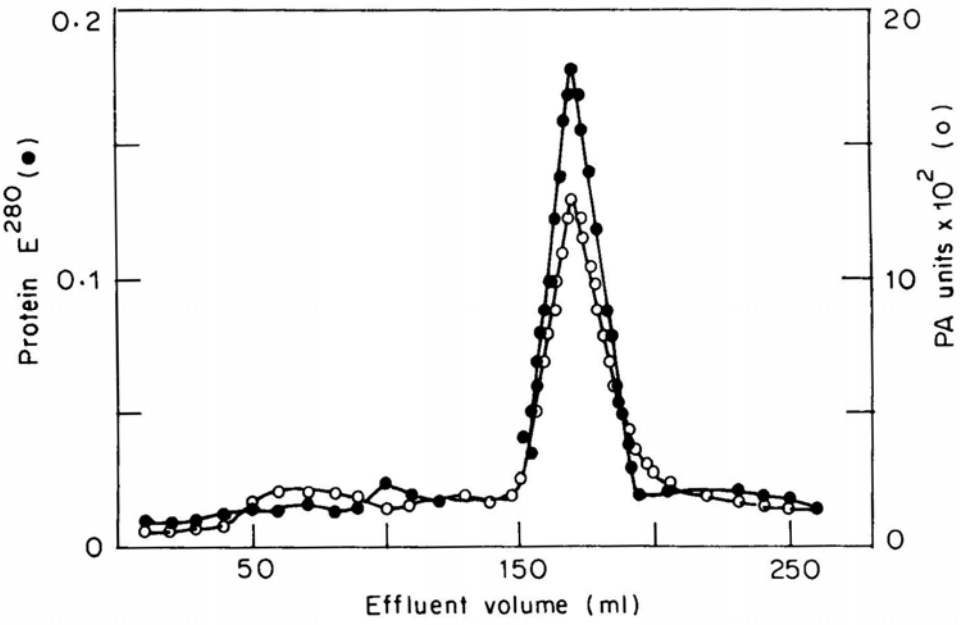

Figure 1. Chromatography in plasminogen activator of lymphosarcoma of ascites on *p*-aminobenzamidine Sepharose.
The clear dialyzed ammonium sulphate precipitated preparation (11·5 ml) was applied to a column of *p*-aminobenzamidine Sepharose equilibrated in 0·05 M Tris-HCl buffer, pH 7·5. After collecting the flow-through of the column (≃3 %. of enzyme activity) the column was washed with the equilibrating buffer. The enzyme was eluted subject to three changes of buffer (≃150 ml) as described in § 2·2. At the time of elution, 1 ml fractions (corresponding to effluent volume 151-200 ml) were collected and 15 μl aliquots were analysed for enzyme activity. The arrow indicates the start of elution in the effluent.

Table 1. Purification of plasminogen activator of lymphosarcoma of ascites (LS-A).

Fraction	Volume (ml)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
20,000 *g* supernatant	108	81·36	62	100 (4962)	1
Ammonium sulphate precipitate	12	38·04	105	80·4 (3994·2)	1·7
p-Amino-benzamidine-Sepharose eluate	25	0·177	9982	35·6 (1776·8)	163

Enzyme activity (PA) is expressed as plasmin units. A unit of plasmin is defined as the amount of enzyme giving rise to an increase of 1×10^3 extinction per min at 280 nm.

by PAGE showed a few major bands in the crude preparations, only one Coomassie blue stainable band was evident in the *p*-aminobenzamidine peak suggesting the apparent homogeneity of the preparation. This experiment was essentially conducted to show the reduction in the number of protein components

Table 2. Biological activity of plasminogen activators.

Enzyme	Assay methods			
	Caseinolytic activity (units/μg)	Amidolytic activity (units/μg)	Fibrinolytic activity (lysis time) (min)	Binding to fibrin (%)
Lymphosarcoma (LS-A)	11·05	0·21	14·0	59·0
Standard t-PA	18·1	0·27	11·5	66·0
Urokinase	17·0	0·25	13·5	0·42

Enzymatic assays were performed as described in § 2·2. Amidolytic activity is expressed as follows. Unit of amidolytic activity = Amount of activity that converts one mol of substrate per second under standard conditions. Per cent binding to fibrin was determined by reading the absorbance (405 nm) following incubation of supernatant with plasminogen and S-2251 for 30 min at 37°C.

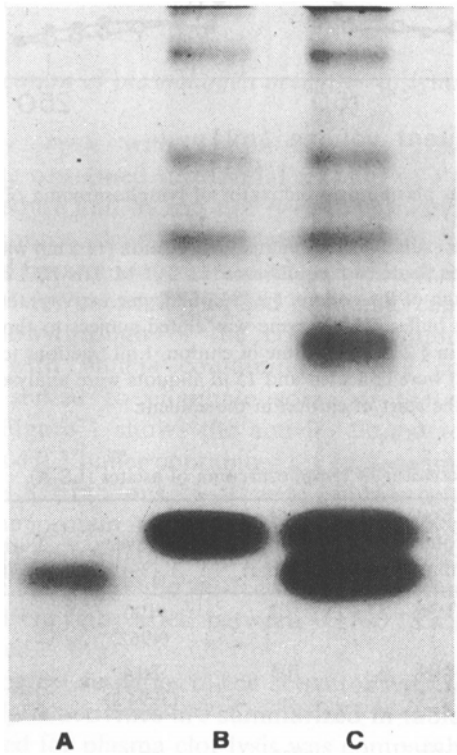

Figure 2. Polyacrylamide slab gel electrophoresis of lymphosarcoma (LS-A) derived plasminogen activator preparations.

Electrophoresis was conducted by the procedure of Lugtenberg *et al* (1975) at 6 mA for 18 h in 7·5% polyacrylamide gels followed by staining with Coomassie blue. **(A)** Dialyzed 20,000 *g* supernatant fraction (100μg); **(B)** ammonium sulphate fractionated extract (100 μg); **(C)** eluate (15 μg) from *p*-aminobenzamidine-Sepharose 4B peak.

during the purification protocol, particularly following the affinity chromatography. The standard protein markers were therefore not run alongside.

Purity of the samples obtained during the stages of purification was also

ascertained by SDS-PAGE at pH 8·3 as shown in figure 3. As a result of purification protocol the number of Coomassie blue-stainable, protein bands were marginally decreased. The stained bands when restained by silver staining procedure (Wray *et al* 1981) did not show any additional sensitivity. The purified preparation of activator in the peak fraction migrated as a single protein band indicating the presence of a single polypeptide chain, the apparent molecular weight of which corresponded to about 66,000 daltons under reducing conditions. Gel filtration of the activator on Sephadex G-100 was also in keeping with this estimated figure derived from Standard calibration curve of several proteins of known molecular weights.

The purity of the activator was further checked by running its HPLC profile. The elution pattern is depicted in figure 4 which shows a single protein peak on HPLC confirming its purity. A comparison of its migration with authentic Standard proteins also revealed an apparent molecular weight of ~ 66,000 daltons.

Immunodiffusion analysis was performed to define the nature of lymphosarcoma activator. Antibodies against this activator were produced in rabbit to examine specificity and immunological cross-reactivity between lymphosarcoma activator, Yoshida sarcoma activator (Nulkar and Pawse 1991), standard t-PA and urokinase.

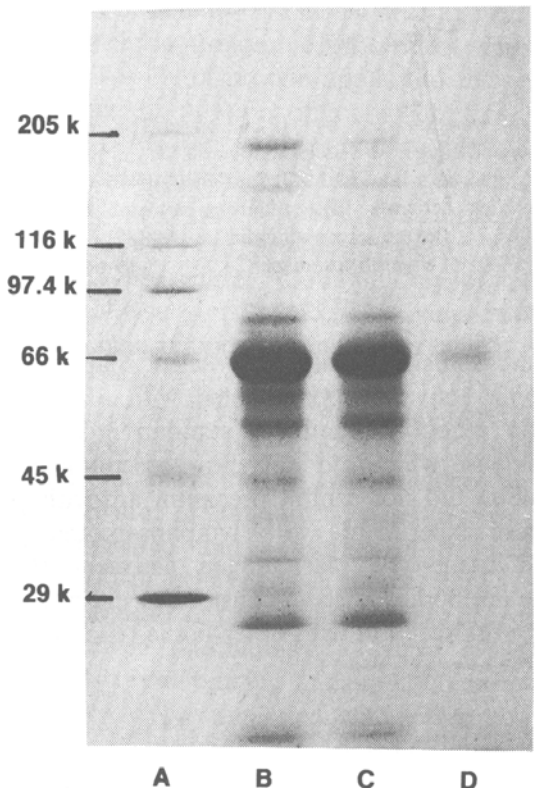

Figure 3. Analysis by SDS-PAGE of activator during stages of purification.

Protein samples were electrophoresed in the presence of 0·1% mercaptoethanol and 0·1% SDS in a 4·5% polyacrylamide gradient gel according to the procedure of Laemmli (1970). **(A)** Standard marker protein (10μg); **(B)** dialyzed 20,000*g* supernatant extract (20μg); **(C)** ammonium sulphate fractionated extract (15μg); **(D)** *p*-aminobenzamidine Sepharose peak material (10 μg).

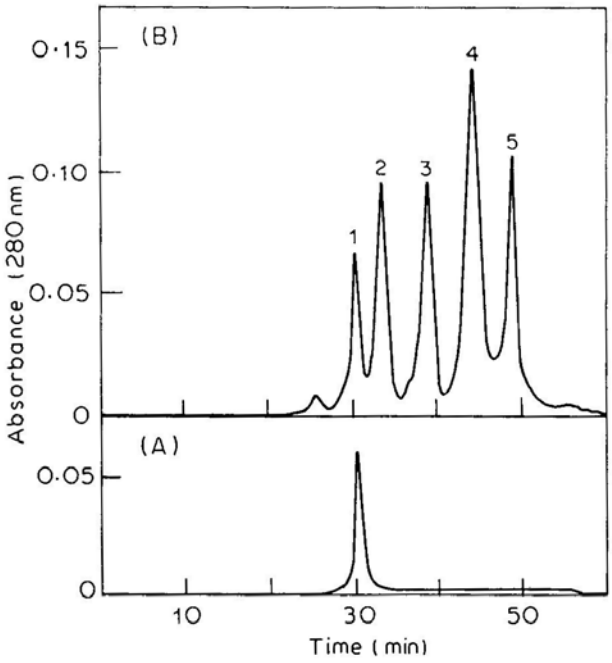

Figure 4. HPLC of t-PA.
Purified LS-A (40 μg) was analysed by HPLC on a TSK G 3000 SW column (0·75 × 60 cm) equilibrated with 50 mM Tris-HCl, 100 mM NaCl, pH 7·2. The same buffer was used to elute the protein at a flow rate of 0·5 ml/min. Absorbance was monitored at 280 nm. **(A)** Lymphosarcoma activator, **(B)** Standard mixture of protein (50 μg each) of known molecular weights. (1) Bovine serum albumin (66,000); (2) ovalbumin (45,000); (3) carbonic anhydrase (29,000); (4) chymotrypsinogen (24,000); (5) cytochrome (12,500).

Ouchterlony's double immuno-diffusion analysis revealed that lymphosarcoma activator and Yoshida sarcoma activator cross-reacted with a rabbit antiserum showing a positive precipitin reaction. Similarly, standard t-PA also reacted positively in contrast to urokinase which did not show precipitin arc (figure 5). Serum of the non-immune rabbit did not exhibit precipitin arc with either of the activators or urokinase. These experiments with lymphosarcoma point to its immunological identity with tissue-type plasminogen activator thus differing distinctly from urokinase. The antibodies raised against LS-A did not appear to be highly specific because of the cross-reaction with other tissue-type PAs.

4. Discussion

The results described above reveal that cells of lymphosarcoma contained plasminogen activating potential. The fibrinolytic activity of this activator was assessed by comparing with standard t-PA and urokinase. Reports concerning the purification procedures of plasminogen activators emphasise the use of affinity matrices such as arginine or lysine, Sepharose 4B, Con A-Sepharose 4B, zinc chelate Sepharose 4B and immunoadsorbents (Kluft *et al* 1983; Wallen *et al* 1983; Einarsson *et al* 1985). In the present work purification procedure for

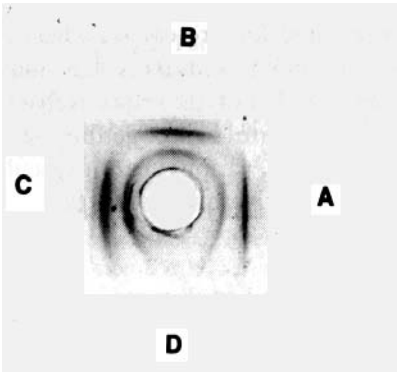

Figure 5. Ouchterlony double immunodiffusion analysis.
Central well, Rabbit antiserum (10 μl from antiserum diluted 4 times with saline). **(A)** Lympho sarcoma activator (20 μg); **(B)** standard tissue plasminogen activator (20 μg); **(C)** Yoshida sarcoma activator (20 μg); **(D)** urokinase (20 μg).

lymphosarcoma activator was devised by affinity chromatography on amino-caproyl *p*-aminobenzamidine-Sepharose 4B avoiding extremes of pH or denaturing conditions. The loss of activity associated with the tumour cells was minimized by avoiding multistep chromatographic procedures. The peak of enzymatic activity contained about 0·3–0·4% of protein applied to the column. The final preparation showed a specific activity of approximately 9982 IU/mg. The activator has a lower specific activity as compared to the Standard t-PA. This may suggest a possible difference in the fibrinolytic potential of this activator and standard t-PA. Variations in specific activities of tissue plasminogen activators in non-malignant cells and a number of cell lines have been reported (6000-90,000 IU/mg) depending on the use of assay procedure. About 0·17 mg of the purified enzyme could be obtained from tumour cells of 12 mice. The lymphosarcoma activator contained a single polypeptide chain of apparent molecular weight of 66,000 daltons as revealed by SDS-PAGE.

Plasminogen activators of variable molecular sizes have been identified by SDS-PAGE technique (Hoal *et al* 1983; Dodd *et al* 1986). The binding studies indicate that the activation of plasminogen gets enhanced in the presence of fibrin by lymphosarcoma activator or standard t-PA but not by urokinase. Immunodiffusion analysis further indicated that antiserum of this activator did not react with urokinase. The activator behaved in a manner similar to Yoshida sarcoma PA in terms of immunological response and other properties. It would seem that lymphosarcoma derived plasminogen activator activity could be of tissue-type origin as judged by fibrin binding and immunological studies. It is necessary to mention that plasma when incubated with either activator or standard t-PA at 37°C for 24 h displayed negligible breakdown of fibrinogen while urokinase induced significant fibrinogenolysis. Implicit in this discussion are the reports of Katzenberger *et al* (1987, 1988) suggesting the presence of urokinase like plasminogen activator in fast growing cells and showing possible implications in the tumour growth. Recently, correlations of plasminogen activator and plasmin-like activities with fibrinolytic activity have been documented in growth of an experimental tumour (Tozser *et al* 1989). It would seem that two types of plasminogen activators displaying variability in their action could exist in different

strains of tumour to generate plasmin required for proteolysis whenever the need arises. A difference in the distribution of u-PA and t-PA has indicated their functional roles in invasive processes and thrombolysis respectively (Collen *et al* 1989). Tissue-type of plasminogen activators are hence getting recognition as effective therapeutic agents for treatment of various cardiovascular diseases (Verstraete 1990). Activity of lymphosarcoma activator remains to be evaluated in respect of fibrin-dissolving potential.

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