PURIFICATION OF HL-A ANTIGENS FROM THE URINE OF DISEASED AND HEALTHY INDIVIDUALS

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1. Introduction

The HL-A antigens used for most of the biochemical studies in the past have been solubilized from cellular materials. However in such cases the recovery of purified active molecules is always low, no matter which solubilization technique is used.

Accordingly, a richer and/or more abundant source of HL-A antigens which can easily be purified had to be sought. Two recent publications have reported such sources: Billing and Terasaki [1] showed that large quantities of HL-A antigens are present in human serum, and Robert et al. [2] were able to demonstrate the presence of HL-A antigens in the urine of patients suffering from tubular proteinuria.

Since urine is abundant and easy to collect, we have partially purified HL-A antigens from that of diseased and of *normal* individuals.

2. Materials and methods

The urine was provided by donors whose HL-A specificities had been phenotyped. Antigenic activity in the urines was monitored by either the lymphocytotoxicity inhibition test [3] or the complement fixation inhibition test [4]. The urines were purified: a) by filtration on Sephadex G-75, G-100 and G-200 in 0.05 M Tris–HCl, 0.15 M NaCl buffer, pH 7.3; b) by chromatography on DEAE-Sephadex A-50 in 0.05 M Tris–HCl, 0.05 M NaCl buffer, pH 8.0. The active molecules were eluted after a continuous gradient was applied: 0.3 M NaCl, mixing chamber of 200 ml; c) by preparative polyacrylamide gel electrophoresis (pH 9.4, 7% acrylamide for the separating gel, 4% acrylamide for the stacking gel), using a Canalco apparatus (PD-2/150 column). Analytical polyacrylamide gel electrophoresis of the purified active fractions was carried out in SDS containing systems [5] and at pH 9.4. Proteins were determined by the Folin procedure of Lowry et al. [6] in concentrated and dialysed fractions (Diaflo-membranes, UM-20, AMICON).

3. Results and discussion

Urine from several diseased and healthy individuals were tested for the presence of HL-A antigens (table 1).

It should be noted that the specificity ratio (SR) in the crude urines tested was very low in each case and simple filtration on Sephadex did not improve the ratio. However, DEAE-Sephadex chromatography enabled a great number of proteins which interfered with the serological tests to be eliminated, for all the HL-A specificities tested after elution from the column had a much higher SR (# 6).

3.1. *Urine from diseased individuals*

Fig. 1 shows the elution profile after ion-exchange chromatography. The two HL-A specificities tested

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Table 1
Characterization of HL-A specificities in the urine of diseased and healthy individuals

Source of urine	Phenotype HL-A	HL-A specificities tested	Protein (mg/l)*	Total ID50 (IU/l)	Specific activity (IU/mg protein)	Specificity ratio (SR)**
Cystinosis D.F.	HL-A1, HL-A 3, W 14, W 27.	HL-A 1, HL-A 3	300	15.000	50	2
Cystinosis G.G.	HL-A 2, HL-A 3, HL-A 7, –.	HL-A 2, HL-A 3, HL-A 7.	570	16.000	28	2
Cystinosis V.T.	HL-A 2, HL-A 5, W 32, W 27.	HL-A 2, HL-A 5.	360	50.000	140	2
Wilson disease M.T.	HL-A 2, HL-A 12, W 29, W 27.	HL-A 2	400	64.000	160	2
normal urine A.D.	HL-A 1, HL-A 9, HL-A 8, –.	HL-A 1, HL-A 9	100	14.000 (HL-A 9)	140	2

* Proteins were determined on 1 litre samples concentrated to 4 ml on Diaflo-membranes, UM-20.
** Specificity ratio = ratio of 1 I.U. (= ID_{50} = 50% inhibition dose) determined in a non specific system, to 1 I.U. determined in a specific system.

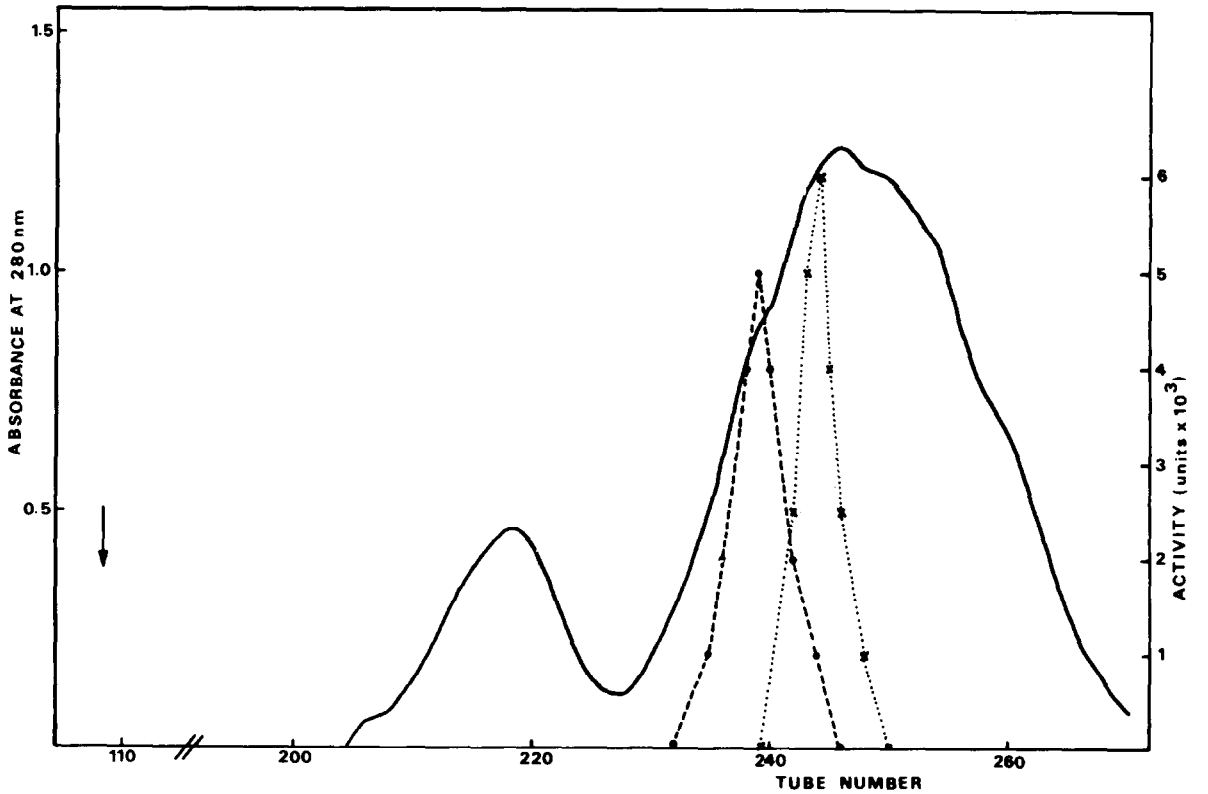

Fig. 1. Chromatography on DEAE-Sephadex A-50 (70 X 2 cm). Sample: 3 liters urine (cystinosis, V.T.), concentrated to 12 ml on Diaflo-membrane UM-20 and dialysed against column buffer (0.05 M Tris–HCl, 0.05 M NaCl, pH 8.0). 3 ml fractions were collected. Application of continuous gradient (0.3 M NaCl, mixing chamber of 200 ml) at fraction 110. The protein elution pattern was determined at 280 nm on 0.1 ml aliquots from each fraction. The inhibitory activity was tested for HL-A2 (- - - -) and for HL-A5 (. . . . .)

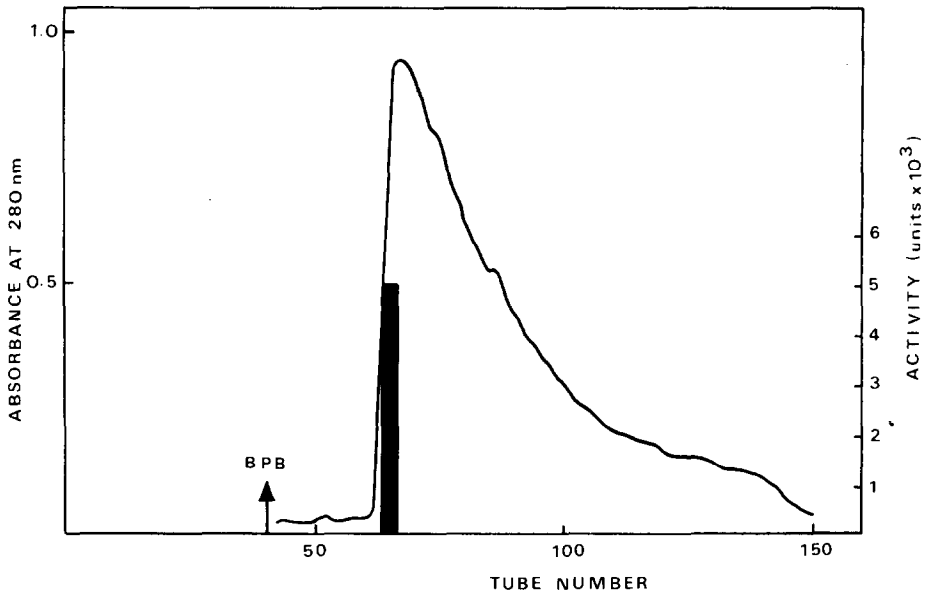

Fig. 2. Preparative polyacrylamide gel electrophoresis of an active fraction eluted from DEAE-Sephadex A-50 (fraction 238 from fig. 1). 4 ml separating gel (7% acrylamide, pH 9.4), 2 ml stacking gel (4% acrylamide, pH 6.7). Electrode buffer: 0.05 M Tris, 0.4 M glycine, pH 8.9; elution buffer: 0.3 M Tris–HCl, pH 8.9. Pre-run until elution of bromophenol blue marker. Electrophoresis at 100 V; 6 mA. 1.5 ml fractions. Elution of HL-A2 activity in fractions 64 and 65 (5.000 I.U.).

were partially separated. The most active fractions were separately submitted to further purification by preparative polyacrylamide gel electrophoresis at pH 9.4. HL-A specificities in a small area could always be seen (fig. 2). Fig. 3 shows the analytical gels of a purified active fraction. Electrophoresis at pH 9.4 gave rise to a single band (stained with amidoblack and with periodic acid-Schiff reagent [7], whereas the active fractions eluted from DEAE-Sephadex all contained several protein bands in the same conditions. After electrophoresis in the presence of SDS, the single band was divided into three constituents of approximately 40 000, 70 000 and 80 000 mol. wt.

It should be emphasised that neither the active fractions purified on DEAE-Sephadex, nor the active fractions obtained after purification by polyacrylamide gel electrophoresis contained beta-2-microglobulin. This may be because of our techniques, but Robert et al. [2] made a similar observation. They were able to demonstrate by serological tests that most of the β-2-microglobulin was separated from the HL-A molecule during purification on DEAE-Sephadex.

3.2. *Urine from healthy individuals*

The preliminary results obtained from the study of normal urine can be resumed as follows: only HL-A 9 with an SR of 2 could be characterized in the crude urine, while other HL-A specificities could not be detected. Billing and Terasaki [1] have already noted this behaviour of HL-A 9 during their studies on human serum. However, after purification on DEAE-Sephadex, the active molecules appeared more monospecific (SR # 6). This was the case for all the specificities detected in the donor, not only for HL-A9. The active molecules in normal urine had the same DEAE-Sephadex and preparative polyacrylamide gel electrophoresis elution profiles as those purified from the urine of diseased individuals.

When the serological activity of the fractions that have been partially purified from urine and that of extracts solubilized from cell membranes is compared, the former appears very low. However, it is possible that the HL-A antigens excreted in urine have undergone some form of inactivation and that in urine the serological activity detected comes from many more

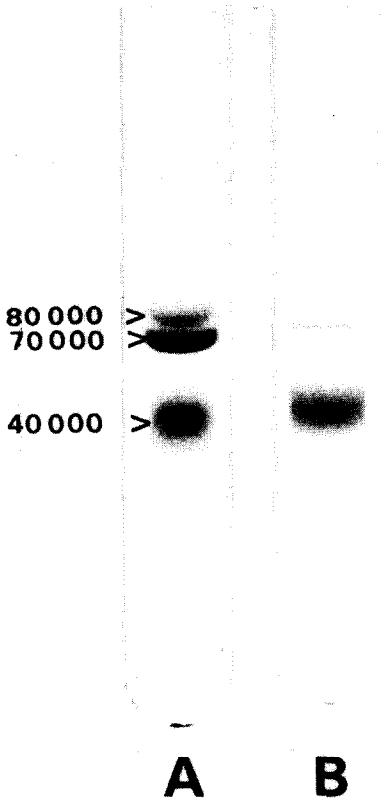

Fig. 3. Analytical SDS acrylamide gels of the active fraction 64 (fig. 2) A. Amido black stained gel. B. Periodic acid–Schiff stained gel.

protein than is the case for HL-A molecules solubilized from cell membranes.

We shall try to determine the activity/protein ratio by further purification of the active molecules, preferably using normal urine which is more abundant and easily available.

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