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ISOLATION OF IMMUNOREACTIVE COMPONENTS FROM EXPERIMENTAL AND HUMAN TUMOUR TISSUES AND SERUMS BY HIGH-PERFORMANCE GEL CHROMATOGRAPHY

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SUMMARY

A method for the rapid separation of proteinous fractions by high-performance gel chromatography was described. Homogenates from tumorous and healthy tissues and blood were eluted by saline on a column packed with rigid hydrophilic macroporous particles of O-glucose—Spheron 300. Fractions were collected and subjected to further analyses. Their antigenic activity was determined by the leucocyte adherence inhibition test method. For the specific immunoactive fractions a dependence of leucocyte adherence inhibition test values on the clinical state of sample donators has been found.

INTRODUCTION

In earlier studies dealing with the problem of separation of fractions possessing the lactate dehydrogenase (LHD) virus and enzymatic (1.1.1.27-L-lactate:NAD oxidoreductase) activity determined in the serum of mice of

various strains [1, 2], the question of application of the gel chromatography of biopolymers was solved by means of a new type of macroporous hydrophilic sorbent based on the copolymer of hydroxyethyl methacrylate and ethylenedimethacrylate, which proved to be satisfactory for the given purpose. In an investigation of other chromatographic applications of these sorbents [3], Borák and Smrž [4], Čech et al. [5], have shown that sorbents exhibit a certain hydrophobic interaction between the copolymer and the lipophilic part of molecules of the compounds under separation. This finding was used to advantage in the papers referred to above, in the separation based on hydrophobic chromatography [6]. The sorption effects, in addition to the molecular sieving properties of Spheron 1000 gel were exploited for the separation of complex, naturally-occurring mixtures of high-molecular-weight compounds like glycosaminoglycans and proteoglycans [7]. If, however, the only effect required from separation is that of the molecular sieve, the hydrophobic interaction of sorbents must be suppressed. This is why, quite recently, highly hydrophilic O-glycosyl derivatives of the basic hydroxyethyl methacrylate carrier have been synthesized and used in the affinity chromatography of lectins [8]. In addition to the function of a specific affinant, the high content of saccharide covalently attached to the internal pore surface (up to 20%, w/w) also causes high hydrophilicity of the carrier and suppresses undesirable hydrophobic sorptions. In this way, a rigid macroporous gel chromatographic material is formed, in which the excellent mechanical and hydrodynamic properties of macroporous Spheron gels are combined with the outstanding interaction characteristics of the Sepharose-type materials used in gel filtration for some time.

In this paper we report a practical application of O-glucose—Spheron in the rather demanding isolation of immunochemically active fractions obtained from homogenates of human and animal tissue and serum, both tumorous and non-tumorous. If a tissue is subjected to a preliminary treatment, consisting of mechanical disintegration of cells, followed by separation of cell fragments and subcellular particles, a clear or slightly opalescent supernatant is obtained, which contains a mixture of proteins and gives positive reaction in the leucocyte adherence inhibition (LAI)-test [9]. Halliday and Miller [9] discovered that in the presence of tumour antigens the adherence of leucocytes to the glass surface is inhibited and derived a semiquantitative evaluation method. Isolation of the active fraction with respect to the LAI-test, its characterization and subsequent treatment still remain an open problem. The method of high-performance gel chromatography provides an advantageous possibility of realization of the first step.

MATERIALS AND METHODS

O-Glycosyl—Spheron 300, a suspension copolymer of 2-hydroxyethyl methacrylate and ethylenedimethacrylate [10] with the exclusion limit of molecular weight 300,000 (tested using dextran standards from Pharmacia, Uppsala, Sweden) was carefully extracted successively with benzene, ethanol and water on a continuous apparatus for 8 h each time, washed with ethanol and ether, and dried. D-Glucose was bonded to the carrier, using an HCl—BF_3

mixture in dry dioxan as catalyst, under conditions described in ref. [6]. The gel, particle size 32—40 μm, was packed in suspension in 0.1 N NaCl solution containing 1% (w/w) of glucose into a column 0.8 × 120 cm and the column was calibrated with dextran standards and a mixture of proteins.

Chromatographic apparatus

The chromatographic equipment consisted of a high-pressure pump MP 2501 (Laboratory Instrument Works, Prague, Czechoslovakia), an injection valve, a column packed with O-glycosyl—Spheron, to which a differential refractometer R 403 (Waters Assoc., Milford, Mass., U.S.A.) and a UV spectrophotometric flow detector with variable wavelength (Optronica, Oberursel/ TS, G.F.R.) were connected in series. The fractions were collected in an LKB Ultrorac 7000 fraction collector (Stockholm, Sweden).

Biological material

Tumorous and healthy tissues and blood were obtained with the kind consent of the Heads of thirteen medical institutes in Prague. The samples under investigation are listed in Table I. Homogenates from biological materials were subjected to centrifugation with cooling at 80,000 g for 30 min. Other methods of the centrifugation of homogenate are discussed elsewhere [11]. Serum samples were obtained by centrifuging blood for 30 min at 4000 g under cooling. The protein level was determined by employing the methods of Lowry and Folin [2].

The activity of E.C. 1.1.1.27-L-lactate:NAD oxidoreductase, was determined using a test kit from Boehringer [12], Mannheim, G.F.R.

Chromatography

A homogeneous supernatant was injected directly into the head of the chromatographic column in an amount of approximately 0.5 ml with a protein content from 3 to 10 mg/ml and eluted with a 0.1 N solution of NaCl containing 1% of glucose at a flow-rate of 100 ml/h at room temperature. The data from the differential refractometer and the spectrophotometric detector were recorded using a two-channel Philips PM 8010 recorder. The 340 nm wavelength used in the UV detection was chosen because it was also used to measure the activity of the above-mentioned isoenzyme. The fractions obtained, 2.5 ml in volume, were again analyzed for the protein content, subjected to the LAI-test [9]. The leucocytes were isolated from heparinized venous blood according to Holáň et al. [13]. After combining this suspension with the proteinous fraction investigated for its content of antigen in the SIAL® test-tubes, free cells were counted in triplicate before and after 2 h incubation at 37°(Bürker chamber). Values higher than 60% of non adhered leucocytes are regarded as positive. The activity of E.C.1.1.1.27-L-lactate:NAD oxidoreductase, was determined by the Boehringer test in all fractions [12].

RESULTS AND DISCUSSION

The effect of modification of the internal surface due to glycosylation is shown in Fig. 1, representing the calibration curve of polydextrans in the case

TABLE I

SAMPLES OF TISSUES AND SERA UNDER INVESTIGATION

Sample*	Diagnosis	
	Benign	Malignant
Human serum	10	11
Mouse serum	12	15
Endometrium	4	5
Cervix	10	11
Ovarium	10	14
Vagina	—	1
Placenta	8	—
Prostate	—	1
Mamma	4	5
Cerebrum	3	3
Cerebellum	1	1
Kidney	1	4
Stomach	—	1
Sigmoid colon	—	2
Lung	4	4
Dental periosteum	1	1
Cutis	1	4 (melanoblastoma)
		3 (psoriasis)
Oculum	1	4 (melanoblastoma)
Os	2	3
Lymph node	2	4 (M. Hodgkin)
Embryonal tissue	8 (1 CEA)	—
Milk	10	—
Others	13	4
Σ	105	101

*Unless otherwise stated samples are of human origin.

of modified and unmodified Spheron 300. Both dependences show that the coating of the internal surface has only a small effect on the exclusion limit and specific pore volume. The covalent bond of glucose, which hydrophilizes the surface, does not influence greatly the molecular weight range of compounds under separation which can penetrate into the gel. An example of a gel chromatogram, where the results of refractometric and spectrophotometric analyses, the protein level after Lowry, the activity of E.C.1.1.1.27-L-lactate:NAD oxidoreductase and the results of the LAI-test have been indicated, is shown in Fig. 2. It provides evidence of the concentration of active components in fractions 12, 13, 14 with a maximum in fraction 13. Chromatographic experiments with detection at various wavelengths have revealed an interesting fact: in most samples of experimental and human tumorous tissues the LAI activity coincides with a maximum absorbancy at 340 nm. There are cases, however (Fig. 3), where the peak from the 13th fraction on the refractometer has no corresponding peak on the spectrophotometer, or in other words, is virtually nil. Such a situation has been observed with operated tumours after complex polychemotherapy, where residual tissue was obtained.

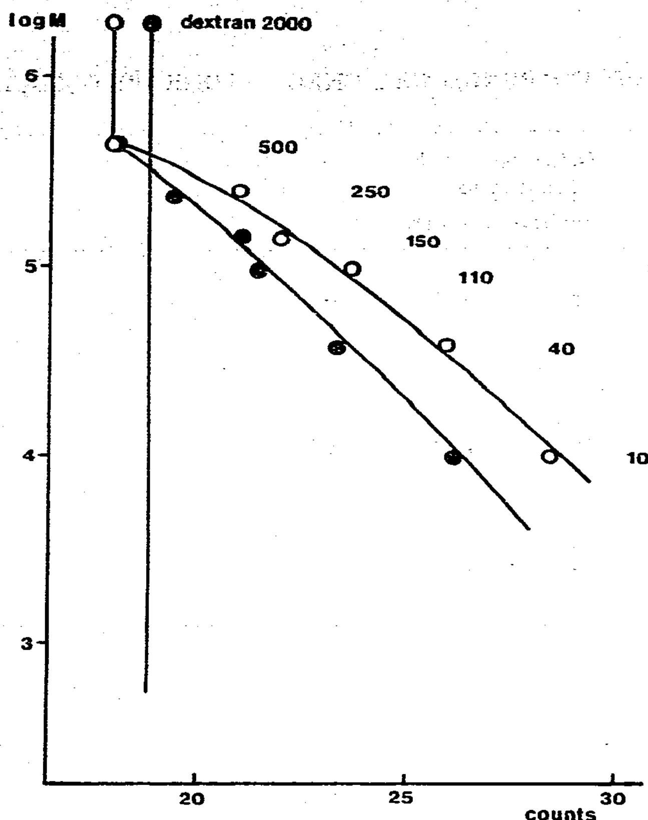

Fig. 1. Calibration curves of Spheron 300 and glucose—Spheron 300. Test mixture: polydextrans of defined molecular weight (M) from 20,000 to 2,000,000 (Pharmacia, Uppsala, Sweden); column: 1200 × 8 mm I.D.; flow-rate: 100 ml/h; eluent 0.1 *N* NaCl containing 1% glucose. ○ = Spheron 300; ● = glucose—Spheron 300.

In any case, the peak detected with the 13th fraction corresponds to the immunoactivity of the sample. These findings are the subject of further study.

A total of 206 separations of experimental and human tumorous tissues and healthy tissues and sera was performed. Proteinaceous fractions from tissues with a maximum UV absorbancy at 340 nm were collected and their antigenic activity was determined by the LAI-test. The correlation between the LAI values and histological findings is summarized, for the most representative groups of tumour diseases, in Table II.

The chromatogram shown in Fig. 4 shows the UV absorbancy and refractive index profiles of a protein fraction with a high content of tumour antigens (human carcinoma cervicis, sample Ce 11).

The method of fast gel chromatography described in this paper permits isolation of protein fractions characterized by a high immunological activity determined by the LAI-test. The activity of these fractions depends on the clinical state of the individual from whom the tissue was taken [9]. A fast and relatively convenient technique allows detection of enriched protein fractions, which are further examined as to the relationship between their

TABLE II

COMPARISON OF HISTOLOGY AND COMBINED GEL CHROMATOGRAPHY—L.A.I. TESTS

Diagnosis (carcinoma)	Number of patients	Histology	L.A.I.-test of the 13th fraction		%
			Positive	Negative	
Cervicis Endometrii Ovarii Vulvae	27	27 positive	25	2	92
Mammae Pulm. Recti Ventric. Others	42	42 positive	40	2	95
Healthy controls	55	55 negative	1	54	98.2

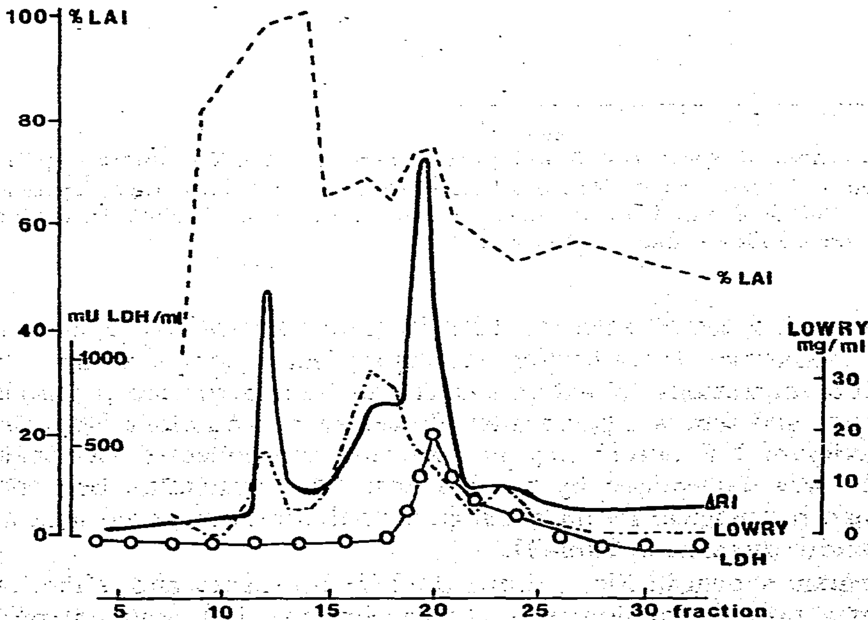

Fig. 2. Gel chromatogram of protein fraction from human carcinoma cervicis (C 13). Conditions: see Fig. 1.

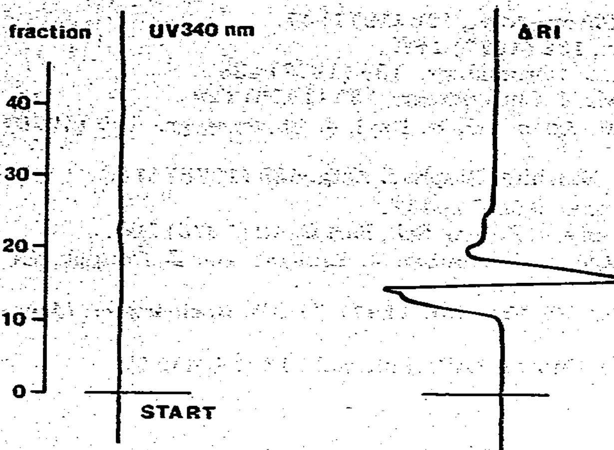

Fig. 3. Gel chromatogram of protein fraction from human carcinoma ovarii after operation and polychemotherapy. Conditions: see Fig. 1.

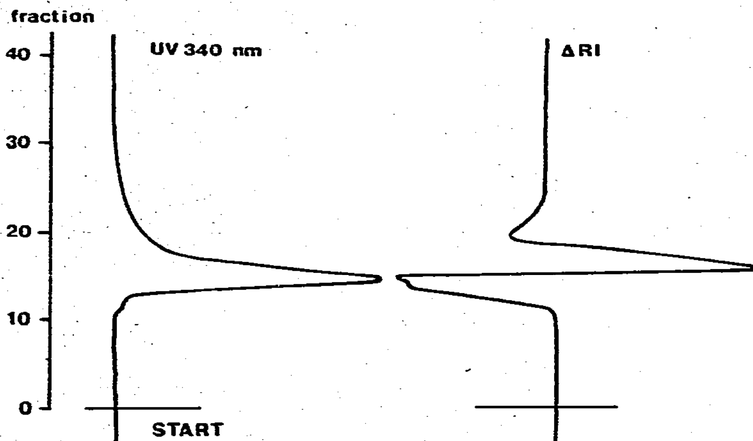

Fig. 4. Gel chromatogram of protein fraction of human carcinoma cervicis (Ce 11). Conditions: see Fig. 1.

composition, biological activity and antigenic character. When comparing the results of the LAI-test of crude homogenates or sera with those of chromatographically separated fractions (Fig. 2) the reliability of LAI confirmed histologically was in the latter case considerably higher.

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