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SEMIPURIFIED HUMAN LEUKOCYTE ULTRAFILTRATE IN HERPES ZOSTER. I. LARGE-SCALE PREPARATION AND BIOCHEMICAL ANALYSIS

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Summary. — Nine batches of lysed human leukocyte ultrafiltrate (LLU) prepared from buffy coats of random healthy donors, as well as their semipurified subfractions — P2/II — were compared in terms of protein, orcinol-reactive material (ORM) content, and ratios of the average values of their ORM and protein contents. Two-step ethanol precipitation and size exclusion chromatography on Sephadex G-15 were used for partial purification and concentration. In comparison to the starting material, approximately 4.4 - fold increase in the ORM/protein ratio of P2/II has been effected. Relatively high variation in both, protein and ORM content of the crude LLU individual batches (ranges: 365 μgs/1 ml — 962 μgs/1 ml; 157 μgs/1 ml — 660 μgs/1 ml, respectively), as well as of those of P2/II fractions (ranges: 16.5 μgs/1 ml — 207.5 μgs/1 ml; 150 μgs/1 ml — 480 μgs/1 ml, respectively) could be observed. The suggested combined purification procedure removed from the LLU about 85 % proteins and 33 % ORM. The removed material contained inhibitors of the cell-mediated immunity (CMI)-inducing and/or augmenting properties of LLU. This is in good agreement with the observed improved therapeutic effect of P2/II fraction in herpes zoster treatment of otherwise noncompromised adults, as described in the companion paper.

Key words: *transfer factor*; *human leukocyte ultrafiltrate*; *large-scale purification*; *size-exclusion chromatography*; *herpes zoster*

Introduction

Since Lawrence's (1976) summation of basic properties and clinical applications of dialysable leukocyte extract (DLE) in many attempts the therapeutical effect of the CMI inducing and/or augmenting properties of DLE

has been investigated in patients suffering from diseases with various underlying immune defects (for reviews see e.g. Khan *et al.*, 1979; Kirkpatrick *et al.*, 1983; Wilson *et al.*, 1987; Fudenberg, 1987; Mayer and Borvák, 1987). In the majority of the cases the therapeutical usefulness of DLE — if properly applied — in human and/or veterinary medicine has been repeatedly reported as outlined recently on the Fifth International Workshop on Transfer Factor (Mayer and Borvák, 1987). Nevertheless, there is also a considerable number of observations with neither unambigous results nor statistically significant correlation between the clinical and immunological data (for reviews see e.g. Kirkpatrick *et al.*, 1983; Mayer and Borvák, 1987). These disagreements probably can be ascribed — among others — to the unforseeable molar ratio of the lysed leukocyte dialysate or ultrafiltrate components (Burger *et al.*, 1976; Nékám *et al.*, 1981; Wilson *et al.*, 1983; Kirkpatrick *et al.*, 1983) to antigen-specific and non-specific CMI effector mechanisms, as well as inhibitory activities (Lawrence and Borkowski, 1983; Holzman *et al.*, 1983; Borkowski and Lawrence, 1983). While the beneficial properties of the LLU component(s) are appreciated in the treatment of viral infections especially on individuals with CMI deficiencies (Carey, 1987; Wilson and Fort, 1987), the presence of inhibitory activities in the preparations is rather undesirable. That's why besides the enrichment attempts (Burger *et al.*, 1983; Paddock *et al.*, 1983; Borvák and Mayer, 1985; Paddock, 1987; Borvák, 1987; Borvák *et al.*, 1987) of the supposed specific soluble CMI inducer (Lawrence, 1955) still greater efforts are focused on the separation of its antagonistic low molecular constituents (Holzman *et al.*, 1983; Lawrence and Borkowski, 1983; Mayer *et al.*, 1983). Three variant molecular forms of the basic oligoribonucleophosphopeptide structure of transfer factor (TF) have been described (Paddock *et al.*, 1983; Paddock, 1987): two cell-associated forms (TFi and TFpre) and one secreted form (TFe). These forms of TF differ either in their internal nucleotide or in the presence of a phosphate on its peptide moiety, probably required for biological activity.

Based on the analogy with the murine model described by Mayer *et al.* (1982, 1983, and 1987) where the lysed splenocyte dialysates from mice immunized with attenuated Langat virus from the tick-borne encephalitis complex showed inducing and inhibitory activities always in two defined peaks obtained by size-exclusion chromatography, we attempted to elaborate a simple procedure resulting in a semipurified subfraction of human LLU with enriched inducing activity (Mayer *et al.*, 1988).

Buffy coats of random healthy donors (containing unpredictable proportionalities of cells bearing the low molecular moieties) served as starting material for a highly reproducible procedure at preparation of final material. The variability of both, protein and purine-bound ribose, i.e. orcinol-reactive material (ORM) contents in the individual batches seems of interest. Despite that the starting material used for ultrafiltration contained always the same amount of mononuclear cells per volume unit, differences were observed in protein as well as ORM content among individual batches.

Materials and Methods

Preparation of LLU. After the leukocyte rich fractions of citrated buffy coats of healthy volunteers have been harvested as described earlier (Borvák and Mayer, 1985), the cell suspension was adjusted with pyrogen-free distilled water so that each of its ml counted 5×10^7 cells (lymphocytes and monocytes), i.e. the amount that corresponds to 0.5 international units of TF. Disruption of the leukocytes and ultrafiltration of the lysate (Amicon ultrafiltration cell, model No. 8400 and DIAFLO ultrafiltration membrane PM 10, Amicon Corp., Danvers Mass. U.S.A.) were performed as described in the previous paper. The lyophilized crude LLU was stored at − 18 °C until further purification. In the presented work 12 batches of pooled buffy coats are described.

Sephadex G-15 fractionation of LLU on analytical and semipreparative columns. Before starting the ethanol precipitation, 200 mg of each of the individual batches of LLU have been passed through an analytical 1.4×90 cm column of Sephadex G-15 (Pharmacia, Uppsala) to make sure that their chromatographic profiles at 260 and 280 nms are similar. The lyophilized ultrafiltrate was dissolved in 2 ml of 0.01 mol.l^{-1} ammonium-bicarbonate. The same solvent was used for descending elution at a flow rate of 7.83 cm.hr^{-1}; 4.4 ml fractions were collected.

On a semipreparative column 2.8×95 cm of Sephadex G-15, 15 ml of 10 % (w/v) solution of lyophilized LLU in 0.01 mol.l^{-1} NH_4HCO_3 was gel-filtered at a flow rate of 7.54 cm.hr^{-1}. Eluent: 0.01 mol.l^{-1} NH_4HCO_3; 4.3 ml fractions were collected and monitored at 260 and 280 nms.

All chromatographic procedures were carried out at 4 °C. Void volume (V_0) of the chromatographic columns used was estimated with Blue Dextran (Pharmacia, Uppsala), the K_{av} and Y values of individual pooled fractions (I − IX) were reported in our previous paper (Borvák and Mayer, 1985).

Two step ethanol precipitation of LLU. The partial purification procedure of the inducing activity carrying species consisted of a double alcohol precipitation (Klesius and Fudenberg, 1977; Mayer *et al.*, 1983) followed by a chromatographic fractionation on a semipreparative column of Sephadex G-15. Briefly: to a given volume (usually 50 − 80 mls) of 10 % (w/v) solution (4 °C cold) of LLU two volumes of chilled 96 % ethanol were added. After standing for 1 hr at 4 °C the fine precipitate (P1) was removed by centrifugation (at 900 g/20 min, 4 °C) and to the supernatant (S1) further two volumes of ethanol were added. After 4 hr at 4 °C the second precipitate (P2) was harvested by centrifugation, dissolved in pyrogen-free distilled water and subjected to gel-permeation chromatography on a Sephadex G-15 column.

Gel-permeation chromatography of P2. The clear pale-yellow 10 % (w/v) solution of P2 in pyrogen-free distilled water was applied to a Sephadex G-15 column equilibrated with 0.01 mol.l^{-1} NH_4HCO_3. For the preliminary chromatography the same analytical column was used as in case of LLU. 1.1 mls of 10 % (w/v) solution of P2 were applied to the column and eluted with 0.01 mol.l^{-1} NH_4HCO_3 at a flow rate of 4.81 cm.hr^{-1} in descending mode; 3.9 ml fractions were collected.

From the same sample 8 ml were applied to the semipreparative Sephadex G-15 column. Descending flow of the eluent at a flow rate of 16.24 cm.hr.$^{-1}$ was used. Collected fractions: 4.8 ml. The effluent was monitored at 260 and 280 nm and the collected fractions were pooled as marked by horizontal bars.

Determination of protein and ORM. The amounts of protein and ORM were estimated by procedure of Lowry *et al.* (1951) and Mejbaum (1939), respectively. Bovine serum albumin and D-ribose were used as standards for construction of the calibration curves.

Sterility control. Sterility during the purification procedure of the harvested material (P2/II), successfully applied in the clinical trial was assured by a thorough clean-down of the cooled off workplace using Persteril (0.3 % (v/v) solution of per-acetic acid) and a 2 hr UV irradiation. All laboratory glass-ware and metal instruments were sterilized by heating up to 200 °C for 2 hr. The sterility of the column effluents and collected fractions of P2/II was checked using common microbiological methods for aerobic and unaerobic flora. Sterile distilled water (Aqua pro injectione, Imuna, n.p. Šarišské Michaľany) was used for preparation of all solutions. All chemicals used were of analytical grade purity.

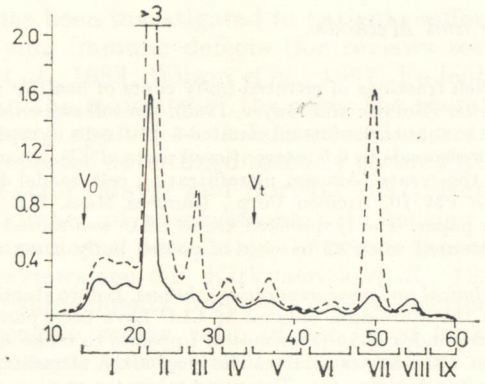

Fig. 1.
Gel filtration on Sephadex G-15 column of lysed human leukocyte ultrafiltrate
About 200 mg LLU was applied at a flow rate of 7.83 cm.hr^{-1} in 0.01 mol/l NH_4HCO_3. I—IX indicate the fractions collected (4.4 ml). Abscissa: tube number; ordinate: absorbance at 260 nm /— — —/ and 280 nm /———/.

Results

Fractionation of LLU on Sephadex G-15

Representative chromatograms of crude LLU on analytical 1.4 × 90 cm column and on semipreparative 2.8 × 95 cm Sephadex G-15 column are shown in Figs 1 and 2, respectively. The preliminary analytical fractionation of 10 batches of LLU before alcohol precipitation, using 0.01 mol.l^{-1} NH_4HCO_3 as eluent gave repeatedly the same elution pattern in terms of elution volume of individual peaks. Although the hinder peaks of LLU

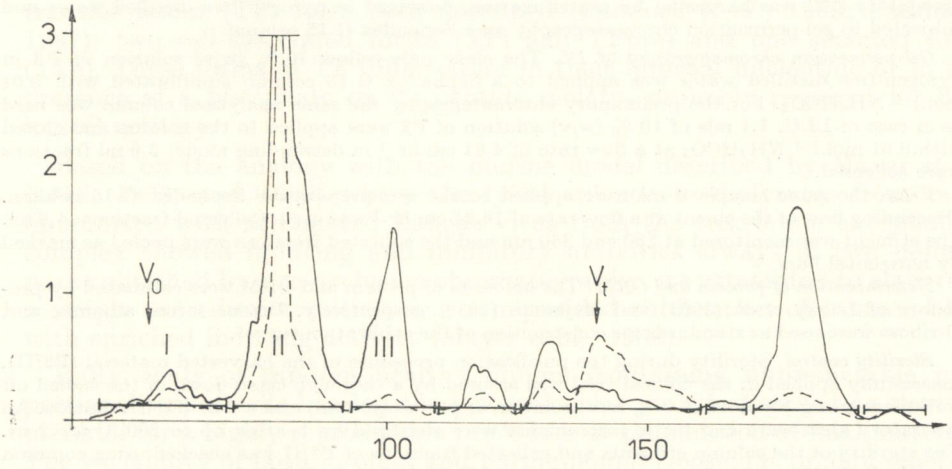

Fig. 2.
Semipreparative purification of LLU on Sephadex G-15 column
For details see Materials and Methods.
Abscissa: tube number; ordinate: absorbance at 280 nm /———/ or 260 nm /— — —/, respectively.

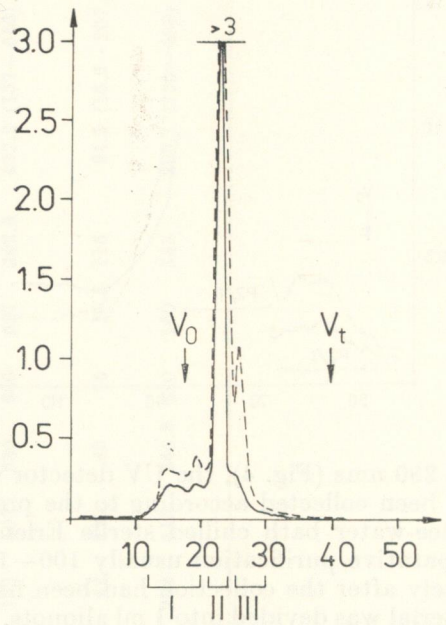

Fig. 3.
Sephadex G-15 gel filtration of the twice precipitated LLU (P2)
Collected fractions (I, II, III): 3.9 ml; flow rate: 4.81 cm.hr^{-1} in 0.01 mol/l NH_4HCO_3.
Abscissa: tube number; ordinate: absorbance at 260 nm /– – –/ or 280 nm /———/.

appeared to adsorb to the gel matrix — as it is common with Sephadex G-15 — good reproducibility was found also while using the semipreparative column.

Gel-permeation chromatography of P2

The chromatographic profile of P2 on analytical and semipreparatived column of Sephadex G-15 is shown on Figs. 3 and 4, respectively. Individual fractions from these separations have been pooled into three sections (marked P2/I, P2/II, and P2/III), where the second of them represents the semipurified final product with supposed inducing/amplifying activity. Although the elution volumes of the corresponding first peaks of LLU and P2 (i.e. peaks I and P2/I, peaks II and P2/II, etc.) are fairly similar (for comparison see Figs. 1—3 and 2—4, respectively), the protein and ORM content of individual batches, and consequently that of their sub-fractions are different. Table 1 shows the protein and ORM content of the LLU batches under investigation, as well as of the semipurified final products — P2/II — derived from them.

Colletion of the final semipurified material for clinical trial

This has been done in a sterilized refrigerator (see Materials and Methods) according to the absorbance profile of P2 monitored at 260 and 280 nms. At the point when the eluate gave rise to an ascending absorbance at 260

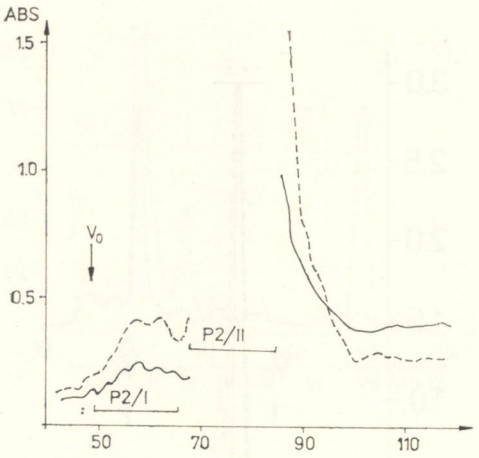

Fig. 4.
Filtration of twice precipitated LLU (P2) on Sephadex G-15 column
Sample: 800 mg P2/8 pyrogen-free distilled water.
Eluant: 0.01 mol/l NH_4HCO_3 at a flow rate of 16.24 cm.hr^{-1}.
Fractions: 4.8 ml
Abscissa: tube number; ordinate: absorbance at 280 nm /———/ or 260 nm /– – –/.

and 280 nms (Fig. 4), the UV detector was ruled out and the P2/II material had been collected according to the previously checked elution volume into an ice-water bath chilled sterile Erlenmeyer-flask. In one course of semi-preparative purification usually 100—120 ml of P2/II were yielded. Immediately after the collection had been finished, the whole volume of the final material was devided into 1 ml aliquots. The filling was accomplished through ethylene-oxide sterilized MILLEX single use filter units (Millipore, 0.42 μm) using sterile syringes in a sterile box. The aliquots have been stored at —20 °C until further use.

Discussion

As pointed out in our previous paper (Borvák and Mayer, 1985), a great number of basic procedures have been used for purification attempts of TF from crude DLE or LLU on analytical scale. However, very few data could be found in this respect for large-scale procedures. Although analytical studies of the LLU's composition and those of their properties are supposed to provide data meaningful for understanding the mechanism of action of TF (Burger *et al.*, 1976 and 1983; Wilson *et al.*, 1983; Lawrence and Borkowski, 1983; Paddock, 1987; Fudenberg, 1987), besides small-pool procedures and single-donor preparations the advantages of a large-scale purification procedure for relatively long-lasting clinical studies are evident (Franke *et al.*, 1987).

In previous studies an ample evidence for the unforeseeable heterogeneity of the low molecular moieties of the leukocyte extract has been given (Wilson *et al.*, 1978; Gottlieb *et al.*, 1979 and 1987; Lawrence and Borkowski, 1983; Fudenberg, 1987). Based on laboratory and clinical studies a two step alcohol precipitation in combination with gel-permeation chromatography proved to be useful for increasing the desirable inducing/augmenting activity of the

Table 1. Comparison of protein and ORM content of individual batches of LLU and their P2/II subfraction

	Batch	A	B	C	D	E	F	G	H	I	J	K	L	Average (Range)
LLU	Protein (μgs/1 ml)	550	770	702	—	—	—	530	490	550	443	365	962	595.7 (365—962)
	ORM (μgs/1 ml)	324	316	291	—	—	—	157	503	630	660	527	392.5	437.2 (157—660)
P2/II	Protein (μgs/1 ml)	—	—	—	112	53	207.5	42	120	80	75	16.5	116	91.3 (16.5—207.5)
	ORM (μgs/1 ml)	—	—	—	252	295.3	150	294	415	246.6	480	160	345	293.1 (150—480)

final material largely devoid of inhibitory substances (Mayer *et al.*, 1983, 1985, 1987, and 1987*a*; Mitrová and Mayer, 1987). Therefore, we focussed our efford to elaborate a simple standardized large-scale purification procedure.

In average, 424 mls of LLU corresponding to 212 TF international units have been processed in one semipurification cycle. The corresponding yield of P2/II material was 110 mls in average, and each of its mls is supposed — according to a formal calculation — to represent such amount of the biological activity (ies)-carrying species that corresponds to approximately 2 I.U. of TF. The biological activity is believed to be connected with the ribonucleotide moiety of the hypothetical oligoribonucleopeptide or oligoribonucleophosphopeptide structure of the TF molecule(s) rather than with its peptide segment. The latter is assumed to be responsible for antigen-specificity (Fudenberg, 1987; Paddock, 1987). If one considers that by the presented semipurification procedure approximately 85 % of the protein content of LLU while only about 33 % of its ORM content had been removed, then the increase in the ORM/protein ratio can be regarded as an empirical indicator of partial purification or copurificatio of activity-carrying entities acheaved. Going on this assumption, our procedure yielded approximately 4.4 - fold concentration [(ORM/prot.) LLU: 0.733 → (ORM/prot.) P2/II: 3.209]. Nevertheless, the success in experimental immunotherapy (i.e. the desired effect on the organism level) with the applied P2/II fraction in herpes zoster cases — as shown in the companion paper (Mayer *et al.*, 1988) — cannot be predicted solely on the basis of these data. It has been shown, however, by means of lymphocyte transformation test that the partially purified P2/II material is superior to crude LLU in an other group of investigated diseases where immune disregulations are known to be involved (Mitrová and Mayer, 1987).

The absence of standardization of *in vitro*, as well as *in vivo* assays for specific inducing and/or augmenting (amplifying) effects on CMI mechanisms of TF, however, still leads to controversial data and brings difficulties to compare the results of assays of individual terms. This is valid mainly for the potent non-specific CMI augmenting or amplifying activity(ies) specified in the case of IMREG materials described by Gottlieb (1987). Despite of the fact that several new tests have been developed in this respect (Marescot *et al.*, 1979; Fudenberg *et al.*, 1983; Gottlieb *et al.*, 1984; Nékám *et al.*, 1987) recently, there is generally still not available sufficient experience with their use. Moreover, due to a great heterogeneity of healthy donors, as well as to a lot of — even minor — differences in the techniques of preparation and purification of individual laboratories, we are not able to compare different batches of TF preparations with sufficient reliability.

One of the simplest approaches how to overcome these difficulties is to get rid of the prevailing majority of low molecular accompanying compounds bearing the specific CMI inducing and augmenting activity(ies) (Figs. 1—3, and 2—4, respectively), as well as to prepare larger, more homogenous batches of LLU and those of P2/II employing the suggested semipurification

procedure. This may prove also as the first step towards the further isolation efforts of the active molecule(s) in a quantity which is required for elucidation of its structure and for the better understanding of the mechanism of its action.

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