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AMINO ACID ANALYSIS OF SELECTED REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC PEAKS OF CRUDE AND PARTIALLY PURIFIED LYSED HUMAN LEUKOCYTE ULTRAFILTRATE

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Summary. — Analytic reversed-phase high performance liquid chromatography (RP-HPLC) was performed to separate from the crude lysed human leukocyte ultrafiltrate (LLU) its partially purified most immunoactive subfraction P2/II *in vivo*. Under conditions used, the highest degree of segregation of both, LLU and P2/II could be observed in the first, as well as in the last two fifth of the water — methanol gradient. The comparison of the RP-HPLC traces of LLU and P2/II suggests that probably some hydrophilic components of LLU have been removed or — at least — diminished. The preliminary amino acid analysis (AA) of the selected peaks showed that none of them lacks Gly, Ser, and Glu. Of the basic amino acid residues Lys has been found with relatively many peaks while hydrophobic as well as aromatic amino acids have been represented very modestly. Further study is warranted in order to determine better the bearings of presented findings for the *in vivo* situation.

Key words: *amino acid analysis*; *RP-HPLC*; *human leukocyte ultrafiltrate*; *transfer factor*

Introduction

Transfer/inducer-factor (TF), unlike to earlier definition (Lawrence, 1955), is at present classified (Chase, 1983; Fudenberg, 1987) as a substance(s) produced by helper lymphocytes (CD4) of the specifically sensitized donor. TF induces in the non-sensitized recipient numerous cell-mediated immune responses in an antigen-dependent manner, regardless of the interspecies barrier. Its unique properties are appreciated in treatment of e.g. viral infections in individuals with immunodeficiences of cellular type (Asher *et al.*, 1976; Klesius, 1981; Kirkpatrick *et al.*, 1983; Mayer *et al.*, 1983; Mayer and

Borvák, 1987; Wilson *et al.*, 1988). As the number of different low molecular mass (<10.000) substances in LLU — the most common source of TF — is considerably high (more than 200 distinct moieties, Fudenberg, 1987), many purification procedures have been attempted to get TF rid of contaminating constituents (Klesius and Fudenberg, 1977; Paddock *et al.*, 1979; Mayer *et al.*, 1983; Borvák, 1987; Huo Bao-Lai *et al.*, 1987; Franke *et al.*, 1987; Borvák *et al.*, 1988). However, until now none of the described purification procedures achieved a resolution power sufficient for production of TF in desired amount and of suitable purity for structural as well as more precise biological analyses.

Our recent contribution, based partially on preliminary experiments (Borvák *et al.*, 1987), *deals* with amino acids *analysis* of selected RP-HPLC peaks of LLU and P2/II. The P2/II fraction administered to patients with herpes zoster (Mayer *et al.*, 1987), as compared with placebo a) provided faster cessation of virus shedding in cutaneous lesions and their accelerated healing; b) induced earlier appearance of and more pronounced leukocyte migration inhibitory factor production.

The nature of the most commonly occuring amino acid residues as well as some parameters of the employed RP-HPLC columns is discussed.

Materials and Methods

Chemicals. The research grade trifluoroacetic acid (TFA) was supplied by Serva (Heidelberg, F.R.G.). Methanol for UV spectroscopy and the other organic solvents were purchased from Merck (Darmstadt, F.R.G.). All other compounds, if otherwise not specified, were of analytical reagent grade (Lachema, Brno, Czechoslovakia).

Preparation of LLU and P2/II has been described elsewhere (Mayer *et al.*, 1983; Borvák and Mayer, 1985).

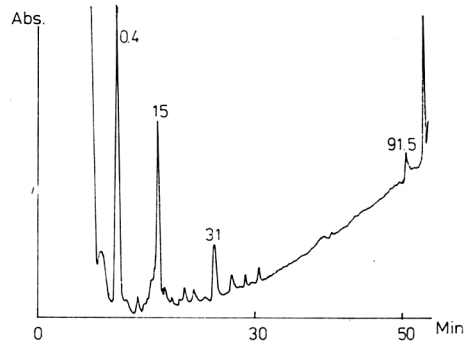
Analytical reversed-phase high performance liquid chromatography. An LKB (Bromma, Sweden) HPLC system with a Shimadzu UV detector SPD-2A was used for all RP-HPLC separations. SGX C-18 column of 250×4 mm I.D. packed with 7 μm particle size Separon (Laboratorní přístroje — Praha) was applied for the separation of either 100 μl or 20 μl aliquots of sample solution. Water and methanol were used as mobile phases to form linear gradient. For degasing of the solvents helium (Gumpoldskirchen, Austria) has been employed. Other conditions of RP-HPLC are described in the legends to the chromatographic figures.

Amino acid analysis. Collected drops corresponding to individual HPLC peaks were vacuum-dried at room temperature, washed with water and vacuum-dried again. The samples were hydrolysed in 6 mol/l^{-1} HCl containing 0.1% phenol at 110 °C for 20 hr in sealed evacuated tubes and subsequently analysed according to Moore and Stein (1954) in a Durrum D-500 amino acid analyzer (Spackman *et al.*, 1958). Norleucine as internal standard has been added to the samples. No special analyses were performed for half-cystine or tryptophan residues.

Results and Discussion

If 100 μl sample aliquots have been applied onto the RP-HPLC column, at least five prominent peaks appeared on analytical RP-HPLC trace of LLU (Fig. 1), the first having been eluted at 0.4% (V/V) methanol concentration, the second at 15% methanol concentration, etc. At the very beginning of the trace a rather hydrophilic peak appears, representing probably a heterogeneous mixture of the ultrafiltrates' components. On the analytical

Fig. 1.
Analytical trace of crude LLU in RP-HPLC
Conditions
Column: Separon SGX C18
Sample: human LLU, 2.5 mg in 100 µl H_2O
Eluant: A. 0.05 % TFA in H_2O
B. 0.05 % TFA in CH_3OH
Gradient: 0% B 5 min., init.
80% B in 40 min., lin.
100% B in 5 min., lin.
Flow rate: 0.5 ml/min.
Temp.: Ambient
Detection: UV at 230 nm, 0.64 AUFS
Abscissa: time (minutes); ordinate: absorbance at 230 nm.

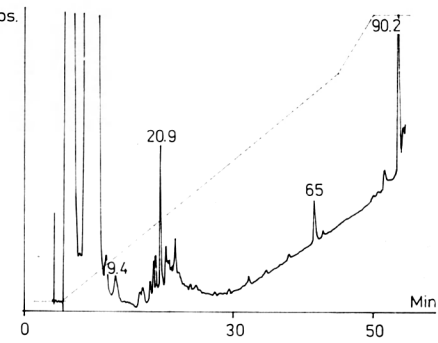


RP-HPLC trace of the semipurified material P2/II (Fig. 2) three fairly pronounced peaks can be seen at methanol concentrations of 20.9%, 65.0%, and 98.2%, respectively. Preliminary analysis showed that the most prevailing common amino acid residues in the first two fractions are Gly and Ser, the first peak including also a great deal of Tyr.

When repeated HPLC analyses with 20 µl sample aliquots were performed, much better resolution could be observed of both, LLU (Fig. 3) and P2/II (Fig. 4). In these cases 26 well segregated peaks were obtained but the entire number of all peaks was larger. Amino acids of the best segregated 9 peaks of LLU as well as that of the best segregated 8 peaks of P2/II are listed in Table 1.

As follows, none of the analysed peaks lacks Gly, Ser, Glu, Asp, Thr, and Ala. As reported by Wilson *et al.* (1977) and Uotila *et al.* (1978), Gly and Ser are the most frequent amino acid residues in the active leukocyte ultrafiltrate subfraction. The third most abundant amino acid residue is Glu which is followed right by Asp, while Thr and Ala were found in much less quantities. Hydrophobic as well as aromatic and basic amino acid residues

Fig. 2.
Analytical RP-HPLC of alcohol precipitated human LLU fraction — P2/II
Conditions
Column: Separon SGX C18
Sample: P2/11, 2.5 mg in 100 µl H_2O
Eluant: A. 0.05% TFA in H_2O
B. 0.05% TFA in CH_3OH
Gradient: 0% B 5 min., init.
80% B in 40 min., lin.
100% B in 5 min., lin.
Flow rate: 0.5 ml/min.
Temp.: Ambient
Detection: UV at 230 nm, 0.64 AUFS
Abscissa: time (minutes); ordinate: absorbance at 230 nm.



were found rarely. No one of the analysed peaks appears to involve Phe, though according to our previous results (Borvák *et al.*, 1987) it has been shown to be present in the dansylation products of non-fractionated LLU as well as in that of P2/II. This disagreement may be ascribed to a relatively high — 44.1% (w/w) — content of free amino acids in the non-fractionated dialysate (Uotila *et al.*, 1978), which need not necessarily appear in HPLC peaks.

HPLC approaches have been described also by Gottlieb (1987) for extraction and purification of cell-mediated immune response amplifiers — materials non-specific with respect to any antigen to which the donor, as well as the recipient were previously exposed — from human leukocyte dialysates. The materials of particular interest are a dipeptide Tyr-Gly, a tri-

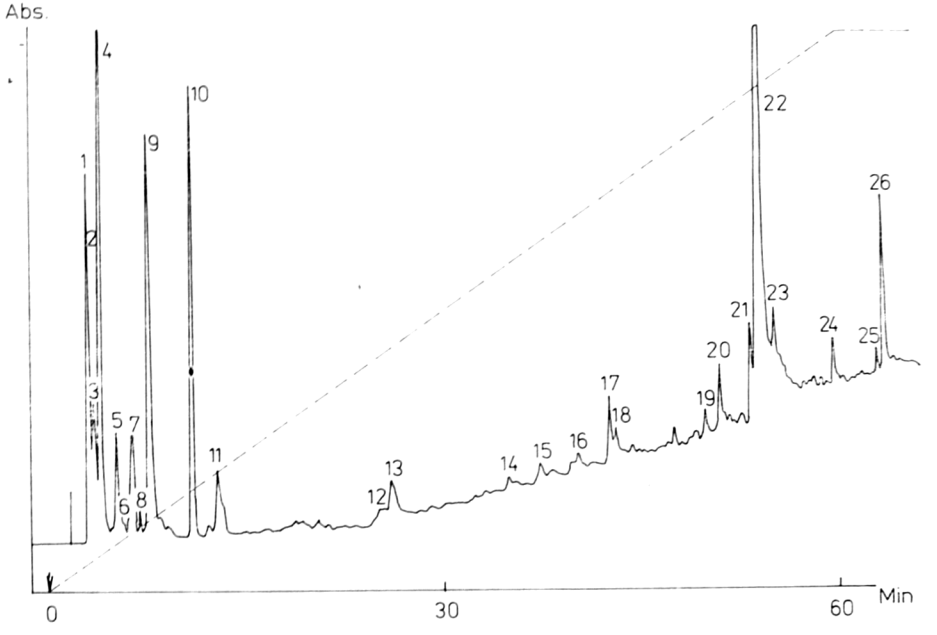


Fig. 3.

Analytical RP-HPLC trace of crude human LLU

Conditions
Column: 250×4 mm I.D.
Separon SGX C18, 7 μm
Sample: 20 μl of LLU
Eluant: A. 0.05% TFA in H_2O
B. 0.05% TFA in CH_3OH
Gradient: 100% B in 60 min., lin.
Flow rate: 0.5 ml/min.
Temp.: Ambient
Detection: UV at 230 nm, 0.64 AUFS
Abscissa: time (minutes); ordinate: absorbance at 230 nm.

peptide Tyr-Gly-Gly, and their derivates. Although this purification process was based on HPLC using a linear gradient of ethanol in water and the parameters of the chromatographic column [octadecylsilane "mu Bondapak" C18, 300×3.9 mm I.D. (Waters Assoc., Milford, Mass.)] were different from ours, the amino acid composition of the first RP-HPLC peak of P2/II, i.e. Gly, Ser, Tyr (Borvák *et al.*, 1987) strongly remembers these amplifiers.

Immune response amplifiers described by Gottlieb (six designated ones) elute at specified parts of the ethanol-in water gradient and can be identified either in terms of the ethanol concentration of the materials coming off the chromatography column, or by the ultraviolet absorption profile (UVAP) characteristics together with the simultaneously monitored retention time. Similarly oriented study of P2/II may yield data about the possible pres-

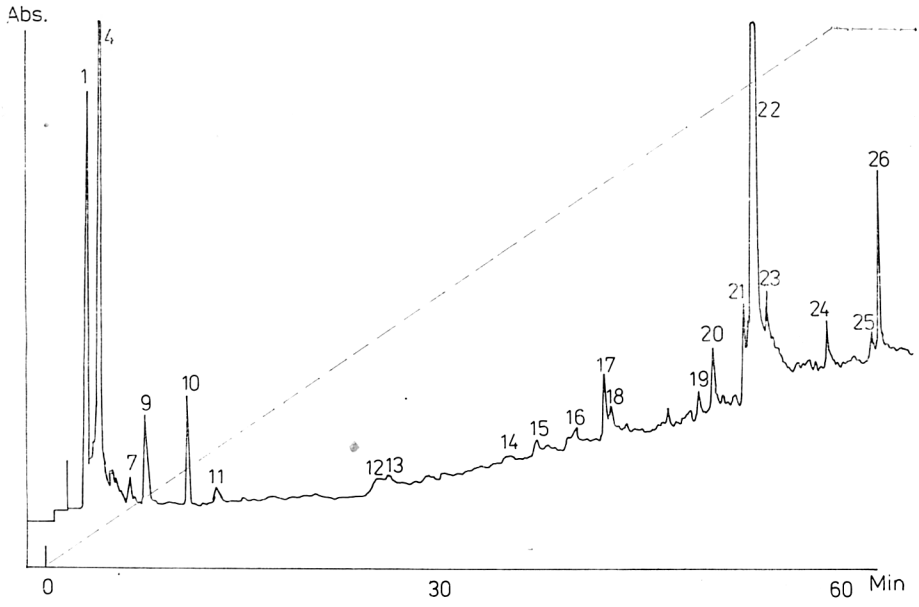


Fig. 4.

Analytical RP-HPLC trace of semipurified human LLU — P2/II

Conditions
Column: 250×4 mm I.D.
Separon SGX C18, 7 μm
Sample: 20 μl of P2/II
Eluant: A. 0.05% TFA in H_2O
B. 0.05% TFA in CH_3OH
Gradient: 100% B in 60 min., lin.
Flow rate: 0.5 ml/min.
Temp.: Ambient
Detection: UV at 230 nm, 0.64 AUFS
Abscissa: time (minutes); ordinate: absorbance at 230 nm.

Table 1. Relative quantities of amino acid residues in the best segregated RP-HPLC peaks of LLU and P2/II

Readed conc. of Methanol (%, V/V)	RP-HPLC peak Nr.	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Lys	His	Arg	Nle
LLU																	
0–1	1	1.7	0.3	1.7	0.9	—	2.7	1.0	0.6	0.1	—	—	0.5	0.2	0.2	1.3	11.7
1–2	2	5.0	1.2	4.9	+	—	+	+	—	—	—	—	—	2.0	—	—	2.6
3	3	1.9	2.2	1.5	10.3	—	3.0	7.2	—	0.2	—	—	—	1.7	—	—	2.5
3–4	4	0.3	0.3	0.6	3.0	3.3	5.3	0.3	2.6	0.2	—	—	—	1.3	1.3	—	—
6–7	7	0.7	0.7	1.8	1.4	0.3	2.0	0.6	0.4	1.0	—	0.2	—	ND	ND	ND	12.3
10	9	—	0.4	1.3	1.0	—	10.6	0.4	—	0.2	—	—	—	0.2	0.3	—	—
16	10	0.6	0.4	1.1	2.1	1.8	7.2	0.6	1.3	0.2	1.7	1.0	—	0.8	0.6	—	13.7
18–20	11	0.7	0.4	1.4	1.0	—	1.5	0.5	—	0.2	0.4	1.7	1.6	0.4	0.2	—	13.6
68	17	0.6	0.4	1.3	1.0	—	1.0	0.4	—	0.2	—	—	—	0.2	—	—	16.9
P2/II																	
0–2	1	0.6	0.4	1.1	1.0	—	1.2	0.4	—	0.2	—	0.2	—	0.3	—	—	12.7
3–5	4	5.6	2.0	4.3	15.5	1.3	12.4	5.1	—	0.2	—	0.2	—	2.4	0.7	—	12.6
10–11	9	0.4	0.3	1.0	0.6	—	6.3	0.3	—	0.2	0.3	—	—	0.2	—	—	11.1
68–69	17	1.0	0.7	1.7	1.5	0.3	1.6	0.8	0.4	0.2	0.2	0.5	—	0.6	0.3	0.3	14.0
82–83	20	1.0	0.7	2.7	1.4	—	2.4	0.9	0.3	0.2	—	0.2	—	0.4	0.3	0.2	11.4
87–88	22	0.8	0.6	2.4	1.0	0.2	2.0	0.8	—	0.2	—	0.2	—	0.4	0.3	—	16.1
97	24	0.5	0.3	1.0	0.8	0.3	1.0	0.3	—	0.3	—	0.2	—	—	—	—	16.1
100	26	0.5	0.3	1.3	0.8	—	1.0	0.3	—	0.2	—	—	—	—	—	—	16.2

+: traces only; ND: not determined; No corrections were made for Tyr, Ser or Thr.

ence of such amplifiers in this material and add to explanation of its marked therapeutical effect as well as throw more light upon their anticipated common structural and/or biological origin and function.

It can be concluded that preparative RP-HPLC seems to be as yet the shortest promissing way of obtaining so called sequentor-grade final products which could contribute to better understanding of the nature of TF.

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