Acta virol. **29** : 119—128, 1985

UV-ABSORBANCE PROFILE OF HUMAN LEUKOCYTIC ULTRAFILTRATE AFTER AFFINITY CHROMATOGRAPHY ON IMMOBILIZED M-AMINOPHENYL BORONIC ACID: IMPLICATION FOR TRANSFER FACTOR PURIFICATION

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Received May 3, 1984

Summary. — The UV-absorbance of the lysed human leukocyte ultrafiltrate (LLU) was investigated by Sephadex G-15 gel permeation chromatography before and after affinity chromatography on immobilized m-aminophenyl boronic acid. This biospecific fractionation caused substantial changes in 260/280-nm absorbance ratio of individual peaks as revealed by Sephadex G-15 gel permeation chromatography. While the material which had passed through the affinity support unretarded (W) appeared to be partly or completely deprived of peak IV, peaks IV and V were fairly enriched in the retarded material (R). This material, on the other hand, nearly completely missed peaks III, VI and VII. Peak III was copiously represented in material W. Boronate affinity chromatography caused enrichment in the protein content as well as in the content of purine/pyrimidine-bound ribose of material R, i.e. cis-diol group containing (RNA-like) material, considered to represent functional transfer factor activity. The described findings further support the use of immobilized derivatives of boronic acid in the separation of coplanar cis-diol group containing compounds from other constituents of LLU.

Key words: boronate affinity chromatography; human leukocyte ultrafiltrate; human transfer factor; purification; gel preparation chromatography

Introduction

The restorative and augmenting activity on cell-mediated arm of the immune response of transfer factor (TF), a low molecular weight (<10 000) antigen-specific moiety among many other substances present in lysed leukocyte dialysates (LLD) or LLU is widely accepted (Kirkpatrick *et al.*, 1983). Its unique properties are appreciated in the treatment of e.g. severe viral infections in individuals with immunodeficiences of cellular type. Therefore, understanding of the nature and mechanism of action of TF advances both immunological (Lawrence and Borkowsky, 1983) and biochemical studies

(Burger *et al.*, 1983; Wilson *et al.*, 1983). Along these approaches emerges the concept of the unique chemical structure of TF possessing a polypeptide component with a free carboxy terminus and a phosphodiester linkage to a moiety with a free 3' hydroxyl. Additional constraints suggest the inclusion of V_H region or a low-molecular fragment of helper T-lymphocyte antigen receptor in the TF structure. As for all these studies, the analysis of physico-chemical and biological properties of the crude or partly purified LLD or LLU can only be of limited value, in the last few years many attempts (Goust *et al.*, 1976; Dunnick and Bach, 1977; Krohn *et al.*, 1977; Wilson *et al.*, 1977; Burger *et al.*, 1979, 1983; Paddock *et al.*, 1983; Mayer *et al.*, 1983, 1983a; Petersen *et al.*, 1983) have been made to purify this immunoactive molecular species to such degree that it should be suitable not only for more precise biological but also for structural studies or, at least for investigations on its chemical composition.

Chromatograms of LLD or LLU have been reported by many authors (Baram and Mosko, 1962; Baram *et al.*, 1966; Arala-Chaves *et al.*, 1967; Gottlieb *et al.*, 1973; Neidhart *et al.*, 1973; Zuckerman *et al.*, 1974; Reymond and Grob, 1975; Goust *et al.*, 1976; Dunnick and Bach, 1977; Krohn *et al.*, 1977; Wilson *et al.*, 1977; Uotila *et al.*, 1978; Schröder and Rovenský, 1982; Mayer *et al.*, 1982, 1983) but the results cannot be compared satisfactorily, since different methods of LLD/LLU preparation and different techniques of chromatography have been applied.

Biospecific chromatography on immobilized derivatives of boronic acid has succesfully been used in isolation and purification of low molecular weight compounds with coplanar cis-diol groups (Weith *et al.*, 1970; McCutchan and Gilham, 1973; Hageman and Kuehn, 1977; Goitain and Parsons, 1978; Annamalai *et al.*, 1979; Olsson, 1979). Boronate derivatives on various solid supports (Weith *et al.*, 1970; Gehrke *et al.*, 1978; Ackerman *et al.*, 1979) have been used in a number of experiments, however, in only two of them (Burger *et al.*, 1983; Paddock *et al.*, 1983) were deliberately applied for purification and investigation of the basic properties of TF.

In this paper we report the effect of boronic affinity chromatography on the 260/280-nm absorbance ratio of individual peaks from Sephadex G-15 gel permeation chromatography of LLU as well as on the ratios between individual peaks of this material. K_{av} values (see Materials and Methods), as well as content of both polypeptides and purine/pyrimidine-bound ribose of individual peaks before and after affinity chromatography are discussed.

Materials and Methods

Preparation of LLU. Human LLU was derived from a pool of 20 citrated buffy coats obtained from the Blood Transfusion Service (City of Bratislava — Public Health Institute) as follows: blood of normal healthy volunteers was centrifuged (Janetzki K 26.4 × 500 ml, 4 °C) at 2000 rev/min for 40 min and the leukocyte-rich fraction was harvested. After further centrifugation of this fraction at 2000 rev/min for 20 min, the leukocytes were disrupted by 10 times freezing (−70 °C, dry ice/ethanol-bath) and thawing (37 °C, water-bath). The lysate adjusted with pyrogen-free distilled water (1 ml corresponded to 5×10^7 disrupted cells) was then centrifuged at 18,000 g for 20 min and the supernatant ultrafiltred through an Amicon PM 10 membrane using a pressure of 3.5 × 101,325 Pa at 4 °C. The ultrafiltrate concentrated by lyophilization for the use in further experiments was stored at −18 °C immediately after freeze-drying or after 48 hr at

Table 1. K_{av}-values, 260/280-nm absorbance ratios (Y), protein and purine/pyrimidine-bound ribose contents of individual fractions from a representative fractionation of LLU, W and R materials on Sephadex G-15

	Fraction number	K_{av}	Y	Protein content (μg/lmg)	Purine/pyrimidine-bound ribose cont. (μg/l mg)
LLU					
	I	0.103	1.992	31.0	0.0
	II	0.336	4.566	32.5	36.0
	III	0.594	5.885	30.5	1.0
	IV	0.801	2.043	*ND	ND
	V	1.034	2.389	ND	ND
	VI	1.370	2.760	ND	ND
	VII	1.602	5.797	ND	ND
	VIII	1.938	0.360	ND	ND
	IX	ND	ND	ND	ND
W					
	II	0.362	3.202	37.0	21.0
	III	0.620	3.924	36.0	0.0
	IV	0.827	1.210	ND	ND
	V	1.034	1.746	ND	ND
	VI	1.395	2.270	ND	ND
	VII	1.628	5.007	ND	ND
	VIII	1.990	0.381	ND	ND
	IX	2.430	2.320	ND	ND
R					
	I	0.103	0.807	300.0	26.0
	II	0.439	0.599	192.0	100.0
	III	0.594	0.707	104.0	16.0
	IV	0.827	2.425	ND	ND
	V	1.085	3.617	ND	ND
	VI	1.421	ND	ND	ND
	VII	1.654	1.500	ND	ND
	VIII	2.016	ND	ND	ND
	IX	2.430	3.931	ND	ND

* not determined because of the low yield of lyophilized materials

room temperature. In the lyophilized ultrafiltrates no significant differences were found concerning either their absorbance profiles at 260 and 280 nm on Sephadex G-15 gel permeation chromatography or the similar ratios between their individual peaks.

Gel permeation chromatography. In a representative run 300 mg of lyophilized LLU were dissolved in 3 ml of 0.01 mol/l NH_4HCO_3 (pH 7.8) and applied to a 1.44×90 cm column of Sephadex G-15 (Pharmacia, Uppsala). 0.01 mol/l ammonium-bicarbonate buffer was used for descending elution at a flow rate of 12.0 cm/hr; 2.3-ml fractions were collected. Column effluent was monitored by reading the absorbance at 260 and 280 nm. All fractionation procedures were conducted at 4 °C. Concentration of the effluent was achieved by lyophilization. Void volume (V_o) of the column used was estimated with Blue Dextran (Pharmacia, Uppsala) and the K_{av} values for different fractions were calculated according to the formula:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$ (V_e, elution volume of a given fraction; V_t, total volume of the packed bed).

Boronate affinity chromatography. Spherical polyacrylamide beads (wet mesh size: 100—200; diameter of hydrated beads: 80—150 μm) with immobilized m-aminophenyl boronic acid (100

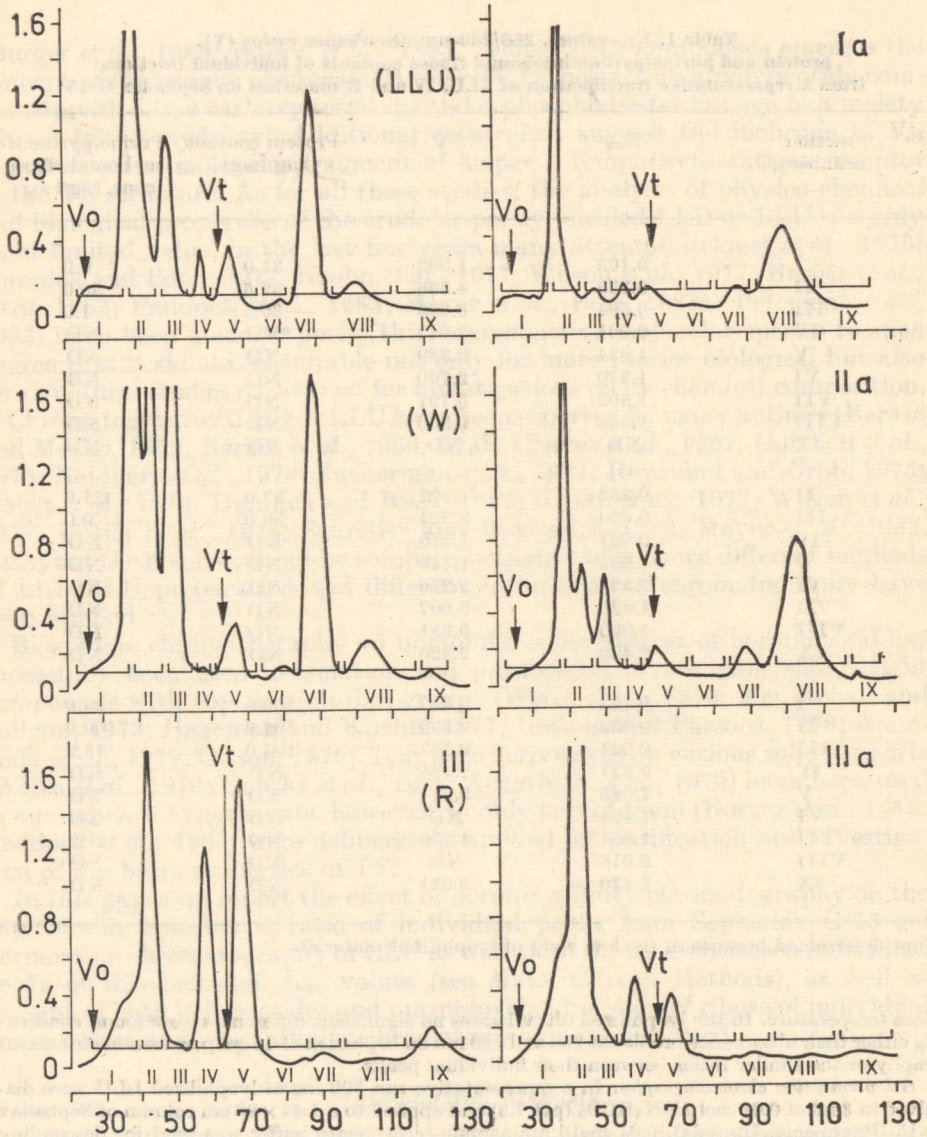

Fig. 1.

Representative chromatography of LLU ,W and R materials (parts I, II and III, respectively) on a 1.44 × 90 cm Sephadex G-15 column

Eluent: 0.01 mol/l ammonium-bicarbonate buffer pH 7.8; flow rate: 12.0 cm/hr; 2.3 ml aliquots were collected and monitored at 260 nm (I, II and III, in the left) and at 280 nm (Ia, IIa and IIIa, in the right).

Abscissa: fraction number; ordinate: absorbance. The effluent was pooled as marked by horizontal bars (I—IX).

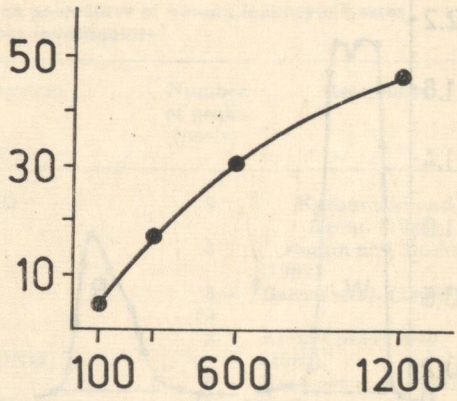

Fig. 2.

Relation of the load applied to the boronate affinity chromatography support (column: 1.05 × 9.50 cm) to the amount of specifically bound cis-diol functionality containing material

Abscissa: amounts (mg) of lyophilized LLU applied to the affinity support; ordinate: amounts of specifically bound cis-diol group containing material [expressed as the weight (mg) of area under the peak specifically released from the affinity support by the change of the pH of the eluent].

μmoles/ml of the settled gel) (Pierce, Rockford, IL U.S.A.) were poured into a 1.05 × 9.50 cm column (V_o = 8.25 ml) as a 50% aqueous slurry containing 0.1 mol/l NaCl and 0.05% NaN_3. The gel was washed with 50 ml of 0.1 mol/l ammonium-acetate buffer pH 5.2 and then with a vol of 0.1 mol/l ammonium-acetate buffer pH 8.45 at a flow rate of 20.0 cm/hr until the pH of the effluent reached 8.45 as well (approx. 10 volumes of the packed bed). At this point different vol. (3—20 ml) of 10% (W/V) sample solution in 0.1 mol/l ammonium-acetate buffer pH 8.45 were applied to the column to test the relation of the load to the amount of specifically bound coplanar cis-diol group containing material. After the sample had soaked into the gel, the unretarded material was washed through the column with 0.1 mol/l ammonium-acetate buffer pH 8.45 at a flow rate of 20 cm/hr until the A_{254} nm of the effluent dropped below 0.05. At this time the elution solvent was changed for 0.1 mol/l mmonium-acetate buffer pH 5.2 and the elution was continued until the second UV-absorbing peak appeared in the effluent. All chromatographic steps were carried out at 4 °C. Fractions of both, unretarded and specifically bound material were pooled according to A_{254} nm, lyophilized and used for gel filtration experiments on Sephadex G-15.

Determination of protein and purine/pyrimidine-bound ribose content. The amounts of protein and purine/pyrimidine-bound ribose of LLU and those of peaks LLU, W and R from Sephadex G-15 chromatography were estimated by the 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.

Results

Fractionation of LLU, W and R on Sephadex G-15

Repeated fractionation of crude LLU materials studied on a single 1.44 × × 90 cm column of Sephadex G-15, using 0.01 mol/l ammonium-bicarbonate pH 7.8 as eluent, gave the same elution pattern. The column effluent was pooled into nine fractions (I—IX) according to the absorbance at 260 and 280 nm. Representative chromatograms obtained from gel filtration of 300 mg of lyophilized LLU, W and R are shown in Fig. 1, parts I, II and III, respectively.

The K_{av} values, 260/280-absorbance ratios (Y), protein and purine/pyrimidine-bound ribose contents of individual peaks (pooled fractions) from representative fractionations of LLU, W and R materials on Sephadex G-15 are given in Table 1. The first four fractions (I—IV) had a K_{av} value of <1.0,

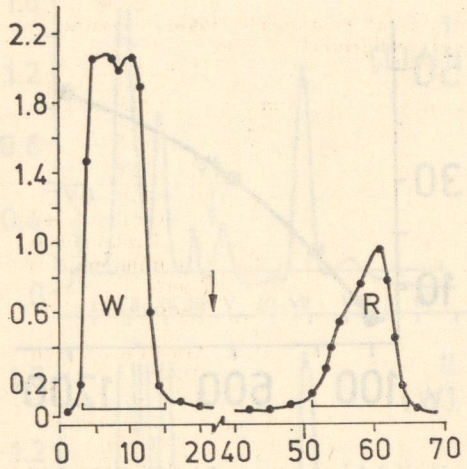

Fig. 3.

Representative boronate affinity chromatography of LLU

600 mg of lyophilized LLU were applied to a 1.05 × 9.50 cm column of immobilized m-aminophenyl boronic acid in 6 ml of 0.1 mol/l ammonium-acetate buffer pH 8.45 at a flow rate of 20.0 cm/hr. Eluents: 0.1 mol/l ammonium-acetate buffer pH 8.45 for washing out the unretarded material and 0.1 mol/l ammonium-acetate buffer pH 5.2 for releasing the specifically bound coplanar cis-diol group containing material; 2.3 ml fractions were collected and monitored at 254 nm.

Abscissa: fraction number; ordinate: absorbance. The effluent was pooled as marked by horizontal bars (W and R, respectively).

while the five subsequent ones adsorbed to the gel matrix and thus had a K_{av} value of <1.0. Fraction V eluted just behind the V_t having a K_{av} value of 1.03.

Boronate affinity chromatography.

Before starting the boronate affinity chromatography, we tested the relation of column load to the amount of specifically bound material. As shown in Fig. 2, the amount of the specifically bound cis-diol group containing material (expressed in terms of the area under the peak specifically eluted from the affinity support) only slightly increased when the load exceeded about 600 mg of lyophilized LLU. A representative boronate affinity chromatography of LLU is shown in Fig. 3. After several runs of biospecific chromatography of LLU (a total of about 2200 mg of lyophilized material) on immobilized m-aminophenyl boronic acid, the unretarded material (W) as well as the specifically bound cis-diol group containing one (R) were pooled, lyophilized and subjected to gel permeation chromatography on Sephadex G-15 column, the conditions of chromatography being the same as for the starting LLU (Fig. 1, part II and III, respectively).

It is evident that the material contained in peak IV is in W nearly totally absent. Peaks IV and V are, on the other hand, fairly enriched in R. Consequently, it can be assumed that the bulk of molecular species in these fractions is coplanar cis-diol group-containing (e.g. RNA-like) material. The material R was nearly completely devoid of peak III and peaks behind the V- from Sephadex G-15 column, however, the ninth peak poorly or even undetectably present in either W or LLU, was markedly enhanced in this material (at about 2V-). Peak III was generously represented in W.

Discussion

Table 2 gives a comparison of several basic procedures used in purification attempt of crude LLU/LLD. As far as the number of peaks is concerned, our

Table 2. Comparison of basic purification procedures of human leukocyte lysates used by various investigators

Mode of preparation	Type of chromatography	Buffer system	Number of peaks (pools)	References
F-T, C, U	Seph. G-10	dist. H_2O	9	Karhumäki and Krohn (1983)
S, C, D	DEAE-cell.	PO_4	3	Baram and Mosko (1962)
F-T, C	Seph. G-200	PO_4	3—	Baram *et al.* (1966)
	Seph. G-25		—10	
S, D	Seph. G-25	H_2O or CH_3COONH_4	2	Arala-Chaves *et al.* (1967)
F-T, D	Seph G-25	PO_4	6	Neidhart *et al.* (1973)
F-T, D	Seph. G-25	NH_4HCO_3	11	Zuckerman *et al.* (1974)
F-T, D	Seph. G-10	NH_4HCO_3	3	Gottlieb *et al.* (1973)
F-T, D	Seph. G-25	NH_4HCO_3	6—	Wilson *et al.* (1977)
	Bio-Gel P-4		—2	
F-T, U	Seph. G-25	NH_4HCO_3	5—	Reymond and Grob
	Seph. G-10		—8	(1975)
S, F-T, C, D	Bio-Gel P-10	dist. H_2O	7	Goust *et al.* (1976)
F-T, C, D	Seph. G-25	PO_4 NaCl	7	Schröder and Rovenský (1982)
C, D	Seph. G-15	—	11	Marescot *et al.* (1979)
F-T, U	Seph. G-15	NH_4HCO_3	9	This communication

Abbreviations used in the Table: F-T: freeze-thawing
C: centrifugation
U: ultrafiltration
D: dialysis
S: sonication

results resemble those of Karhumäki and Krohn (1983) and Reymond and Grob (1975). In the former case 9 peaks, in the latter 8 ones were reported. Concerning chromatographic media for TF purification, Sephadex G-15 only has been used up to now (Marescot *et al.*, 1979). Although the number of pooled fractions as well as their chemical characteristics obtained by these authors differ from ours, Sephadex G-15 — with respect to the complexity of the purified material (Lawrence and Borkowsky, 1983) as well as to the good mechanical parameters of the gel — still remains the medium of choice. As far as the 260/280-nm absorbance ratio (Y) of individual peaks of LLU is concerned (Table 1), boronate affinity chromatography caused substantial changes in their values. Of particular interest is that the Y was enhanced in the IVth and Vth fractions from material R and slightly in fraction VIII of material W only (by 18.69%, 51.40%, and 5.83%, respectively). This phenomenon is thought to be connected either with the enrichment of 260 nm-absorbing material or with decrease of the material absorbing at 280 nm, or, at the same time, with the both of these changes in the fractions in question. On the other hand, nearly

sixty per cent decrease in Y of fraction I, and more than an 86 per cent decrease in Y of fraction I, and more than an 86 per cent decrease in Y of fractions II and III of R material has been found. This finding is well consistent with a general increase in polypeptide content of these fractions. More moderate (34.6% in average) changes have been observed with fractions II, III and IV of W (Table 1). Peaks IV and V of R (Fig. 1, part III) are, at the same time (according to the specificity of the affinity support), assumed to be enriched in cis-diol group containing (e.g. RNA-like) material.

Orcinol test was nearly exclusively negative in fractions I and III of LLU and in those of W, and only weakly positive in fraction II of LLU and in that of W, as well as in fractions I and III of R. A strong increase in orcinol-reactive material content was observed in the second fraction of R material. There was a general increase in the polypeptide content of the first three fractions of R. Our results (concerning LLU) are comparable with those obtained by Reymond and Grob (1975), Marescot *et al.* (1979) and Mayer *et al.* (1982), although the separation media of gel permeation chromatography and/or the way of pooling of chromatographic fractions were completely different. Till now, however, there have not been any data in the literature dealing with the protein or purine/pyrimidine bound ribose content of biospecifically purified LLU (i.e. W and R).

Goust *et al.* (1976) have reported that the bulk (75—90%) of RNA-like material as well as the biologically most active components of chromatographically separated crude LLD on Bio-Gel P-10 (exclusion limit: 20 000 Da) eluted shortly after the void volume. Similar results were obtained by Reymond and Grob (1975), Gallin and Kirkpatrick (1974) and Arala-Chaves *et al.* (1974), all using Sephadex G-25, but contradicting ones by Neidhart *et al.* (1973), Wilson *et al.* (1977), Baram *et al.* (1966), Krohn *et al.* (1977), LoBuglio *et al.* (1973) and Burger *et al.* (1979).

Even if our present findings do not deal with any of the biological activities contained in LLU/LLD or in its biospecifically separated subfractions, they seem to be in good agreement with the results reported by Burger *et al.* (1983). These authors succeeded in 6 experiments in binding TF activity to immobilized m-aminophenyl boronic acid having thus confirmed the presence of a cis-diol (ribose) functionality in TF. This technique may provide a useful preparative tool for separating cis-diol group containing compounds from other constituents of LLU or its chromatographically purified subfractions. Studies on this topic are in progress.

References

Ackerman, S., Cool, B., and Furth, J. (1979): Removal of DNA from RNA by chromatography on acetylated N-;N′-(m-dihydroxylborylphenyl)succinamyl: aminoethyl cellulose. *Anal. Biochem.* **100,** 174—178.

Annamalai, A. E., Pal, P. K., and Colman, R. F. (1979): Purification of nucleosidyl peptides by chromatography on dihydroxyboryl-substituted polyacrylamide and cellulose. *Anal. Biochem.* **99,** 85—91.

Arala-Chaves, M. O. ,Lebaco, E. G., and Heremans, J. F. (1967): Fractionation of human leukocyte extracts transferring delayed hypersensitivity to tuberculin. *Int. Arch. Allergy appl. Immunol.* **31,** 353—356.

Arala-Chaves, M., Ramos, M. T. F., Rosado, R., and Branco, P. (1974): Transfer factor 'in vitro' Additional data concerning a new method. *Int. Arch. Allergy* **46,** 612–618.

Baram, P., and Mosko, M. M. (1962): Chromatography of the human tuberculin delayed-type hypersensitivity transfer factor. *J. Allergy* **33,** 489–506.

Baram, P., Yuan, L., and Mosko, M. M. (1966): Studies on the transfer of human delayed-type hypersensitivity. *J. Immunol.* **97,** 47–420.

Burger, D. R., Vandenbark, A., Vetto, R. M., and Klesius, P. (1983): Human transfer factor: specificity and structural models, p. 33. In C. H. Kirkpatrick, D. R. Burger and H. S. Lawrence (Eds): *Immunobiology of Transfer Factor*, Academic Press, New York.

Burger, D. R., Wampler, P. A., Vandenbark, A. A., and Regan, D. H. (1979): A structural model for human transfer factor. *Discussion paper. Ann. N. Y. Aca.d Sci.* **332,** 236–240.

Dunnick, W. A., and Bach, F. H. (1977): Specificity and structural analysis of guinea pig transfer factor-like activity. *J. Immunol.* **118,** 1944–1950.

Gallin, J. I., and Kirkpatrick, C. H. (1974): Chemotactic activity in dialyzable factor. *Proc. natn. Acad. Sci. U.S.A.* **71,** 498.

Gehrke, C. W., Kuo, K. C., Davis, G. E., Suits, R. D., Waalkes, T. P., and Borek, E. (1978): Quantitative high-performance liquid chromatography of nucleosides in biological materials. *J. Chromatogr.* **150,** 445–476.

Goitein, R. K., and Parson, S. M. (1978): Rapid separation of 5-phospho-α-D-ribose-1-diphosphate from its metabolic derivatives. *Anal. Biochem.* **87,** 636–640.

Gottlieb, A. A., Foster, L. G., and Waldman, S. R. (1973): 'What is transfer factor?' *Lancet* **2,** 822–823.

Goust, J. M., Marescot, M. R., Lesourd, B., Doumercq, S., and Moulias, R. (1976): Characterization of human dialyzable transfer from normal and chronic lymphoid leukemia sources. *Biomedicine* **24,** 39–45.

Hageman, J. H., and Kuehn, G. D. (1977): Assay of adenylate cyclase by use of polyacrylamide-boronate gel columns. *Anal. Biochem.* **80,** 547–554.

Karhumäki, E., and Krohn, K. J. E. (1983): Effect of dialyzable leukocyte extract and other tissue dialysates on Listeria resistance, p. 143. In C. H. Kirkpatrick, D. R. Burger, and H. S. Lawrence (Eds): *Immunobiology of Transfer Fyctor*, Academic Press, New York.

Kirkpatrick, C. H., Burger, D. R., and Lawrence, H. S. (1983): *Immunobiology of Transfer Factor*. Academic Press, New York.

Krohn, K., Uotila, A. Väisänen, J., and Gröhn, P. (1977): Studies on the chemical composition and biological properties of transfer factor. *Z. Immun.-Forsch.* **153,** 395–411.

Lawrence, H. S., and Borkowsky, W. (1983): A new basis for the immunoregulatory activities of transfer factor — An Arcane dialect in the language of cells. *Cell. Immunol.* **82,** 102–116.

LoBuglio, A. F., Neidhart, J. A., Hilberg, R. W., Metz, E. N., and Balcerzak, S. P. (1973): The effect of transfer factor therapy on tumor immunity in alveolar soft part sarcoma. *Cell. Immunol.* **7,** 159–165.

Lowry, O. H., Rosenbrough, J. N., Farr, A. L., and Randall, R. J. (1951): Protein measurement with Folin phenol reagent. *J. biol. Chem.* **193,** 265–275.

Marescot, M., R., Lesourd, B., Moulias, R., Hainaut, J., and Saint-Blancard, J. (1979): Quantification and biological assays of transfer factor, p. 151. In A. Khan, C. H. Kirkpatrick and N. O. Hill (Eds): *Immune Regulators in Transfer Factor*, Academic Press, New York.

Mayer, V., Gajdošová, E., Valášková, M., Gombošová, A., and Oravec, C. (1982): Dialysable specific transfer factor in mice immunized with attenuated Langat virus from the tick-borne encephalitis complex: generation, action and quantitative assay. *Acta virol.* **26,** 453–465.

Mayer, V., Valášková, M., Gajdošová, E., and Oravec, C. (1983): Concentration of murine antigen-specific transfer factor of defined potency. *Acta virol.* **27,** 228–237.

Mayer, V., Gajdošová, E., Mitrová, E., Valášková, M., and Oravec, C. (1983a): Antigen-specific transfer factor induced in mice by an attenuated virus from the tick-borne encephalitis complex: biological, histological, and concentration studies, p. 175. In C. H. Kirkpatrick, D. R. Burger and H. S. Lawrence (Eds): *Immunobiology of Transfer Factor* and H. S. Lawrence (Eds): *Immunobiology of Transfer Factor*, Academic Press, New York.

McCutchan, T. F., and Gilham, P. T. (1973): Studies on polynucleotides containing hybrid sequences. Synthesis of oligonucleotides containing thymidine, adenosine and single deoxyribonucleotidyl-(3′-5′)-ribonucleotide linkage. *Biochemistry* **12,** 4840–4846.

Mejbaum, W. (1939): Über die Bestimmung kleiner Pentos emengen insbesondere in Derivativen der Adenylsäure. *Z. Physiol. Chem.* **258,** 117–129.

Neidhart, J. A., Schwartz, R. S., Hurtubise, P. E., Murphy, S. G., Metz, E. N., Balcerzak, S. P., and LoBuglio, A. F. (1973): Transfer factor: Isolation of a biologically active component. *Cell. Immunol.* **9**, 319—323.

Olsson, R. A. (1979): Phenyl/dihydroboryl/polyacrylamide beads for chromatography of ribonucleosides in tissue extracts. *J. Chromatogr.* **176**, 239—241.

Paddock, G. V., Wilsson, G. B., Williams, A. M., and Fudenberg, H. H. (1983): Human transfer factor: exogenous labelling, purification and role of ribonucleic acid segment, p. 51. In C. H. Kirkpatrick, D. R. Burger and H. S. Lawrence (Eds): *Immunobiology of Transfer Factor*, Academic Press, New York.

Petersen, E. A., Greenberg, L. E., Manzara, T., Rozzo, S. J., and Kirkpatrick, C. H. (1983): Selective removal of transfer factor acticity with antigen, p. 65. In C. H. Kirkpatrick, D. R. Burger and H. S. Lawrence (Eds): *Immunobiology of Transfer Factor*, Academic Press, New York.

Reymond, J.-F., and Grob, P. J. (1975): Transfer factor preparations: some physico-chemical and biological properties. *Vox. Sang.* **29**, 338—351.

Schröder, I., and Rovenský, J. (1982): In vitro determination of effects in dialysable leukocyte extracts containing transfer factor activity. *Allerg. Immunopath.* **10**, 171—176.

Uotila, A., Marnela, K.-M., Hamblin, A., and Krohn, K. (1978): Human transfer factor in vitro: Identification of L-serine and glycine as components with augmenting effect in lymphocyte transformation assay. *Scand. J. Immunol.* **7**, 495—502.

Zuckerman, K. S., Neidhart, J. A., Balcerzak, S. P., and LoBuglio, A. (1974): Immunologic specifity of transfer factor. *J. clin. Invest.* **54**, 997—1000.

Weith, H. L., Wiebers, J. L., and Gilham, P. T. (1970): Synthesis of cellulose derivatives containing the dihydroxyboryl group and a study of their capacity to form specific complexes with sugars and nucleic acid components. *Biochemistry* **9**, 4396—4401.

Wilson, G. B., Welch, T. M., Knapp, D. R., Horsmanheimo, A., and Fudenberg, H. H. (1977): Characterization of T-, an active subfraction of human dialysable transfer factor; I. Identification of the major component in TF-, a precursor of T-, as hypoxanthine. *Clin. Immunol. Immunopathol* **8**, 551—568.

Wilson, G. B., Fudenberg, H. H., Paddock, G. V., Tsang, K. Y., Williams, A. M., and Floyd, E. (1983): Mechanism(s) of action of human transfer factor: Insights obtained from studying 'Antigen-Liberated Transfer Factor' specific for tuberculin, p. 331. In C. H. Kirkpatrick, D. R. Burger and H. S. Lawrence (Eds): *Immunobiology of Transfer Factor*, Academic Press, New York.