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Purification of immunomodulatory factors in human peripheral blood leukocytes

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ABSTRACT

Procedure is described for purifying low-molecular-weight factors with antigen-aspecific properties from a dialysate of human leukocyte extract. It includes gel chromatography on Sephadex G-25 and G-15, ion-exchange chromatography, reversed-phase high-performance liquid chromatography (HPLC) on a C_{18} hydrophobic column and gel permeation HPLC. The immunosuppressive factor (mol.wt. 800–1000) was purified to near homogeneity. It is probably of peptidic nature, although it is pronase resistant. The enhancer factor (mol.wt. 300–600) is eluted from chromatographic columns together with a hypoxanthine-like substance. Nevertheless, the biological activity cannot be attributed to the purine derivative. Identification of this amplifier activity is still lacking.

INTRODUCTION

A number of immunomodulators from human leukocytes have been described that either enhance or suppress immune functions[1–10]. The characterization of these factors and their reproduction by synthesis is of practical importance in view of their potential use *in vivo* in various clinical disorders and after tissue and organ transplantation.

The presence in leukocyte extracts of *in vitro* and *in vivo* immunosuppressive and immunostimulating activity has been reported previously[3,4,7,11]. This paper deals with the isolation of some of these molecules from peripheral blood leukocytes that exhibit activity on *in vitro* lymphocyte transformation to phytohaemagglutinin (PHA).

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EXPERIMENTAL

Preparation of the leukocyte extract

The leukocyte extract was obtained according to the method of Reymond and Grob[12]. Briefly, ten buffy-coats, containing 40% mononuclear cells and 60% polymorphs, represented the starting material for the purification of the immunosuppressor and enhancing factor. They were obtained from units of 450 ml of blood, collected in plastic bags in citrate dextrose from regular blood donors and separated from red blood cells and platelet-rich plasma after centrifugation of the blood unit at 400 *g* for 15 min at 22°C. After three washes in saline solution, the packed leukocytes were disrupted by adding ten volumes of distilled water, followed by twelve freezing–thawing cycles.

Cellular debris was discarded and the leukocyte extract was ultrafiltered through membranes (molecular weight cut-off 10 000 dalton) (Sartorius, Göttingen, F.R.G.). The leukocyte extract obtained was designatedd DLE (dialysate leukocyte extract). One unit was arbitrarily defined as the material prepared from 10^9 leukocytes (40% mononuclear, 60% polymorphonuclear).

PHA-induced lymphocyte transformation

The assay was performed as described previously[13,14]. Human peripheral blood lymphocytes used for the test were obtained as follows. A 10-ml volume of blood was drawn by venipuncture from healthy donors and defibrinated at 37°C in silica-coated tubes containing polystyrene granules. The lymphocytes were isolated in a gradient of Ficoll-Urovison (1.077 g/ml density) (Flow Labs., McLean, VA, U.S.A.) layering 7 ml of defibrinated blood, diluted 1:4 with Hanks' balanced salt solution (HBSS) (Flow Labs.) onto 3 ml of gradient solution and centrifuging at 400 *g* for 30 min. The cell suspension removed from the interface was washed three times with HBSS and suspended at a concentration of 10^5 cells/ml in RPMI-1640 (Flow Labs.), supplemented with 0.20 m*M* N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid) (HEPES), penicillin (100 IU/ml), streptomycin (50 μg/ml) and 10% serum AB. Lymphocyte transformation was obtained in round-bottomed microtitre tissue-culture plates with increasing concentrations of PHA (0.1–10 μg/ml) (Flow Labs.) and various concentrations of DLE or its fractions from the purification steps. The plates were incubated in a 37°C humidified atmosphere for 72 h. Eight hours before the end of incubation the cells were labelled with 1 μCi of [^{3}H]thymidine (2 Ci/mmol; Amersham, Aylesbury, U.K.) per culture, collected on filters and the radioactivity was measured in 10 ml of Picofluor (Packard, Meriden, CT, U.S.A.) in a scintillation spectrometer.

Purification procedures and analytical methods

All reagents and solvents were of analytical-reagent grade form Merck (Darmstadt, F.R.G.). Methanol and the other solvents were of LiChrosolv grade from Merck. Sephadex G-15 and Sephadex G-25 Super Fine were obtained from Pharmacia LKB (Uppsala, Sweden), Dowex 50W-X8 (H^+) (50–100 mesh) from BDH (Poole, U.K.), Dowex 1-X2 (Cl^-) (50–100 mesh) from Bio-Rad Labs. (Richmond, CA, U.S.A.); μBondapak C_{18} column (300 × 39 mm I.D.) from Millipore–Waters (Milford, MA, U.S.A.); LiChrosorb RP-18 (10 μm) column (250 × 4 mm I.D.) from

Merck and TSK 3000 SW Spherogel column (600 × 7.5 mm I.D.) from Beckman (Altex Division, San Ramon, CA, U.S.A.). The efficiency of the columns was monitored using gel permeation (GP) and reversed-phase (RP) high-performance liquid chromatographic (HPLC) peptide standards, obtained from Synthetic Peptides (Department of Biochemistry, University of Alberta, Alberta, Canada). The data from size-exclusion HPLC were analysed using the Autolab Software GPC/PC program (Spectra-Physics, San Jose, CA, U.S.A.). Synthetic peptides were obtained from Peninsula Labs. (Europe) (Merseyside, U.K.) and bradykinin (triacetate salt) and bovine insulin chain B oxidized from Sigma (St. Louis, MO, U.S.A.).

Dialysis was performed at 5°C, using wet cellulose dialysis tubing with a molecular weight cut-off of 1000 dalton[a] (Spectrum 6, 38 mm; Medical Industries, Los Angeles, CA, U.S.A.). The enzymatic digestion with pronase (Boehringer, Mannheim, F.R.G.) (enzyme: substrate ratio from 1:20 to 1:100) was performed in 0.05 *M* ammonium acetate (pH 8.0) at 37°C. At different times, the samples were acidified with 1% trifluoroacetic acid, boiled for 1 min, centrifuged and then injected into the RP-HPLC column. The degradation with xanthine oxidase (XOD) (xanthine:oxygen oxidoreductase, EC 1.2.3.2) (X4500, Sigma) was performed at room temperature in 0.005 *M* phosphate buffer at pH 7.0[15]; the reaction products were analysed by RP-HPLC using the μBondapak column.

Total acid hydrolysis was performed as previously described[16]. The Edman degradation was carried out in an automatic version using a gas-phase sequencer (Applied Biosystems, Foster City, CA, U.S.A.)[16]. The *o*-phthalaldehyde (OPA) derivatives and the 9-fluorenylmethyloxycarbonyl chloride (FMOC) derivatives of the amino acids were separated on Knauer OPA or Knauer FMOC columns (250 × 4 mm I.D.) respectively, according to the method of Kamp[17]. Protein was determined by the method of Lowry *et al.*[18] with bovine serum albumin (Sigma) as a reference standard; in the presence of very small amounts, the quantification was carried out by amino acid analysis.

Instrumentation

HPLC was carried out using the modular Applied Biosystems apparatus, consisting of two pumps (Model 400 solvent-delivery system), two pulse dampers Model LP-21-LO-Pulse (Scientific Systems, State College, PA, U.S.A.), a Spectraflow 491 dynamic mixer/Rheodyne sample injector, a Model 900 programmable fluorescence detector, a Model 1000S diode-array detector (version 1.0A), a derivatizer/autosampler (Knauer, Berlin, F.R.G.), a high-temperature oven with temperature control unit (Waters–Millipore) and an Applied Biosystems 490 A data processor connected to an Epson FX-800 printer.

The eluents were degassed in an Ultrasonic Degass-Branson 2200 (Branson Europe, Soest, The Netherlands). Before injection, the samples were centrifuged in a Biofuge A from Heraeus Sepatech (Am Kalkberg, Osterode, F.R.G.). A single-path UV-1 monitor control unit and optical unit (Pharmacia, Uppsala, Sweden) were employed for monitoring the effluent from chromatography on open columns; a sample cell with a path length of 1.0 mm was used.

[a] The synthetic peptide physalaemin (mol.wt. 1265 dalton) was recovered in the retentate, confirming the cut-off value.

RESULTS

Purification

Gel chromatography of the starting material on Sephadex G-25 (Fig. 1) was useful for the separation of the two biological activities. In fact, the leukocyte immunosuppressor factor (LsF) is eluted from the column with V_e/V_t (elution volume/total column volume) = 0.5–0.6 (mol. wt. *ca.* 800–1000 dalton) and the leukocyte enhancer factor (LeF) with V_e/V_t = 1.2 (mol. wt. *ca.* 300–600 dalton).

In each experiment an aliquot corresponding to 10 units (0.66 g of lyophilized material) was dissolved in 4 ml of 0.05 *M* ammonium hydrogencarbonate and applied to the column. The active pools were lyophilized to yield a dry residue containing the biological activities.

The purification on Sephadex G-25 could be replaced by chromatography on Dowex 50W-X8 (Fig. 2), using distilled water as eluent; 1 *M* ammonia solution was employed for recovery of the enhancer activity.

The LsF from Sephadex G-25 was fractionated on a column of Sephadex G-15 and then purified by gel and reversed-phase HPLC. The last RP-HPLC trace (corresponding to step vi in Table I) showed a single sharp peak at a retention time of 4.60 min. The UV spectrum of this fraction did not show any significant absorbance at 280 nm. The first-order derivative of the chromatographic peak had a shape "resembling

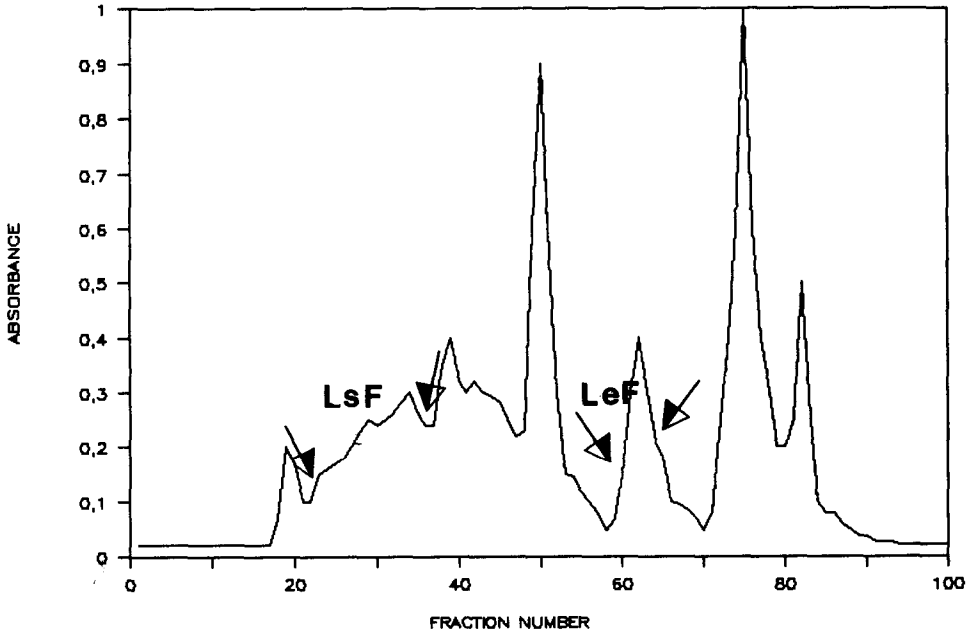

Fig. 1. Gel chromatography on Sephadex G-25 of the dialysate of the leukocyte extract. Column, 1000 mm × 26 mm I.D., stored at 15°C; eluent, 0.05 *M* ammonium hydrogencarbonate; flow-rate, 60 ml/h (one fraction in 10 min); detection, 280 nm (UV-1 sensitivity, 1); recorder, 20 mV, chart speed, 0.1 mm/min; sample size, 10 units. The arrows indicate the pooled fractions containing the LsF and LeF activities, which were subsequently rechromatographed.

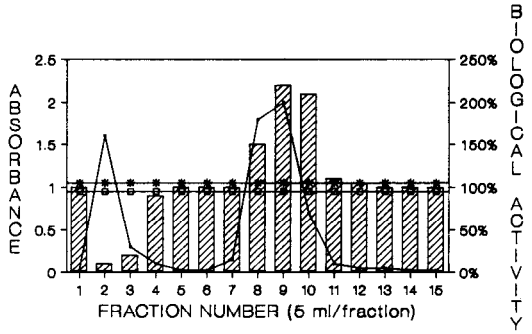

Fig. 2. Ion-exchange chromatography on a Dowex 50W-X8 (H^+) column of the dialysate of the leukocyte extract. Column, 100 mm × 10 mm I.D.; eluents, distilled water (25 ml) and then 1 *M* ammonium hydroxide; flow-rate, 1 ml/min; detection, 280 nm (UV-1 sensitivity, 2); recorder, 20 mV; chart speed, 2 mm/min; sample size, 2 units. The hatched areas represent the biological activity of the fractions; the area between ★——★ and □——□ corresponds to the PHA effects on human leukocytes without the addition of the examined fraction and is considered the reference value. ■——■ = absorbance.

a single cycle of a sine wave" (Users' Manual, Applied Biosystems), indicating that this sample was probably pure.

The immunostimulating activity from the Sephadex G-25 (Table II) or Dowex columns was directly purified by RP-HPLC. In the first HPLC purification step the biological activity was located in the region of the chromatogram between 8 and 10 min. When the collected material was rechromatographed on the same column, LeF activity was associated with the peak with a retention time of 8.89 min. By comparison with a synthetic sample of hypoxanthine, the natural LeF and the synthetic

TABLE I

RECOVERY OF THE IMMUNOSUPPRESSIVE ACTIVITY DURING THE PURIFICATION PROCEDURE

Gel chromatography on Sephadex G-25; conditions as Fig. 1. Gel chromatography on Sephadex G-15: column, 1000 mm × 16 mm I.D., stored at 15°C; eluent, 0.05 *M* ammonium hydrogencarbonate; flow-rate, 10 ml/h; detection, 280 nm. GP-HPLC: column, Spherogel TSK (SW 3000); eluent (isocratic): 0.01% acetic acid; flow-rate, 1 ml/min; detection, 220 nm. RP-HPLC (steps iv and v): column, LiChrosorb RP-18; eluent (isocratic), 0.05% trifluoroacetic acid; flow-rate, 1 ml/min; detection, 220 nm. RP-HPLC (step vi): column, μBondapak C_{18}; eluent (isocratic), 0.005 *M* ammonium acetate; flow-rate, 1 ml/min; detection, 220 nm. ND = Not determined.

Step	*Lyophilized material (LM) (g)*	*Proteins (P) (g)*	*Biological activity*		
			U	*U/LM*	*U/P*
Starting material	13.2	4.400	200	15	45
Sephadex G-25 (step i) active pool	6.7	0.960	200	30	208
Sephadex G-15 (step ii) active pool	1.6	0.155	100	62	645
GP-HPLC (step iii) active pool	0.2	0.015	50	250	3334
RP-HPLC (steps iv and v) active pool	0.01	0.003	50	5000	16 667
RP-HPLC (step vi) active pool	ND	0.0001	25	ND	250 000

TABLE II

RECOVERY OF ENHANCER ACTIVITY DURING THE PURIFICATION PROCEDURE

Gel chromatography on Sephadex G-25: conditions as Fig. 1. RP-HPLC: column, μBondapak C_{18}; eluent A, 0.1% trifluoroacetic acid; eluent B, acetonitrile–water (80:20) containing 0.085% trifluoroacetic acid; flow-rate, 1 ml/min; detection, 220 nm; gradient elution, 1–5 min, 100% eluent A; 6–20 min, 80% eluent B; 21–23 min, 80% eluent B; 24–26 min, 100% eluent B; 27–29 min, 100% eluent B; 30 min, 100% eluent A. The enhancer activity corresponds to the amount (μg) of lyophilized material necessary to produce a 50% increase in lymphocyte response to PHA at various concentrations of the material tested.

Step	*Lyophilized material (g)*	*Proteins (g)*	*Biological activity (μg)*
Starting material	13.2	4.400	516
Sephadex G-25 (step i) active pool	0.078	0.016	50
RP-HPLC (step ii) active pool	0.002	ND	2

purine derivative were unresolved in our HPLC system; in addition, they showed the same UV spectrum (with a maximum at 251 nm at pH 2.7) and identical derivatives. Their sensitivity to total acid hydrolysis and their amino acid analyses appeared to be very similar (see below).

The recoveries of the biological activities attributed to LsF and LeF are reported in Tables I and II.

Preliminary characterization

Enzymatic treatment. Both activities were completely resistant to treatment with pronase. The hypoxanthine-like molecule present in the LeF preparation and hypoxanthine were transformed by xanthine oxidase. The new UV-absorbing peak detected in the RP-HPLC elution profile showed (at pH 2.7) two characteristic maxima in the UV spectrum at 232 (or 231) and 286 nm. The biological activity attributed to LeF was resistant to XOD treatment.

Molecular weight determination. LsF and LeF were linked to chemical entities with molecular weights < 1000 dalton, as deduced by dialysis experiments. In fact, all the material capable of enhancing or inhibiting the response to PHA-treated lymphocytes was in the dialysate.

By size-exclusion HPLC the immunosuppressor showed a molecular weight of *ca.* 1000 dalton and the enhancer activity 300–600 dalton in accordance with the values obtained from chromatography on the Sephadex G-25 column calibrated with synthetic peptides. Nevertheless, these data should be treated with caution. In fact, the elution order of small molecules is not always correlated with their respective molecular weights, probably as a consequence (i) of aspecific interactions between the solutes and the stationary phase and (ii) of the effect of the mobile phase (with reference in particular to the pH values and salt concentrations) on the tertiary structure of the molecules.

The LsF sample obtained by GP-HPLC showed a specific biological activity five times greater than that after Sephadex G-15 (Table I); consequently, this purification step was included in our protocol. The LeF sample obtained in the same way did not show any increase in its specific activity.

Charge detection

The immunosuppressive activity was not retained on a cation exchanger (Dowex 50W-X8, H^+) (Fig. 2) and it co-eluted with an acid molecule, whereas the enhancer factor remained linked to Dowex 50W-X8, because of its basic character, and it was eluted with 1 *M* ammonia solution. The opposite results were obtained by chromatography on an anion-exchange resin (Dowex 1-X2, CH_3COO^-).

Amino acid analysis and primary structure determination

The deduced amino acid composition of the LsF sample with a specific activity of 250 000 A units/g protein after total acid hydrolysis, was 1 Asp, 1 Glu, 1 Ser, 1 Thr, 1 Ala, 1 Gly and 1 Arg. When LsF was analysed by the Edman degradation method, no phenylthiohydantoin derivative could be identified; this indicated that the N-terminus of the peptide material present in the sample was blocked.

The amino acid analysis of the hydrolysed sample of enhancer factor from RP-HPLC showed a peak of glycine and traces of lysine. We assume glycine to originate from the degradation of the hypoxanthine-like molecule present in the sample. In fact, an equivalent amount of hypoxanthine showed the same result, but in this instance the lysine peak was absent[a].

DISCUSSION

Two biological activities have been separated from the DLE obtained by human leukocytes, using gel filtration on a Sephadex G-25 column or ion-exchange chromatography on Dowex 50W-X8. These activities can be defined as antigen-independent, considering the bioassay employed[b]. The former possesses inhibitory properties and the latter enhancing activities (both *in vitro* and *in vivo*)[1–11]. The immunosuppressive agent appears to correspond to a chemical entity with an apparent molecular weight of *ca.* 1000 dalton.

The purification step on a Spherosyl SW 3000 column is useful for obtaining a product with an increase in the specific activity (417% with respect to TsF obtained by Sephadex G-15). Using 0.01% acetic acid as mobile phase, TsF elutes from the Spherosyl column before the peak due to a 0.1-*M* solution of sodium citrate (which is present in the crude starting material). Consequently, the detected biological activity is not due to an aspecific effect induced by salts.

The dificulty in purifying the immunosuppressive factor arises from its properties as follows: (i) LsF is very hydrophilic and does not bind tightly to a C_{18} reversed-phase column; therefore, elution was performed isocratically with water (containing 0.05% trifluoroacetic acid) or a solution of ammonium acetate; and (ii) the factor must be concentrated by lyophilization or evaporation because it is lost by either dialysis or ultrafiltration using the membranes with the smallest pore size commercially available. This is the main reason for the use of volatile buffers as mobile phases in our chromatographic systems.

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[a] A different oxypurine analogue, xanthine, was resistant to hydrolysis with 6 *M* hydrochloric acid (110°C, 15 h).

[b] In the crude DLE there are also antigen-dependent activities able to transfer the delayed-type hypersensitivity (transfer factors).

LsF was purified 5500-fold; the recovery of the biological activity is 25% (Table I). It appears sufficiently pure as judged by the UV spectra of the single peak in RP-HPLC. Considering the behaviour in ion-exchange chromatography and RP-HPLC, the immunosuppressive activity is linked to an acid molecule with very hydrophilic character. A preliminary amino acid analysis has shown that the preparation contains peptidic material, with the N-terminus blocked; nevertheless, the biological activity is resistant to pronase treatment. This result does not exclude the possible identification of LsF with an amino acid chain. The tetrapeptide recently isolated by Lenfant *et al.*[9] and dermorphins[19] are examples of active natural peptides resistant to proteases treatment. LsF structure analyses, now in progress, will clarify this problem, which is unsolved at present.

The suppressive activity cannot be attributed to cell death, as about 90% of the cells in culture were still viable at the end of the incubation with the suppressive factor. Further, (i) the effect is reversible when cells incubated with the suppressive factor are washed before the addition of PHA and (ii) the factor has to be added within 24 h of the addition of PHA in order to manifest the suppressive effect, suggesting that its action is essentially restricted to the early phase of the cell cycle, after the activation process.

It has been reported[5,6] that the immunosuppressive factor is able to inhibit DNA and RNA synthesis on isolated nuclei and in a cell-free system. The effect on cells and on DNA polymerization is dose dependent.

The data suggest that this factor may act directly at the level of DNA polymerization processes with a mechanism similar to those of cyclosporin A[20], although an indirect route through intracellular mediators cannot be excluded[21].

The apparent molecular weight of the enhancer factor is the range 300–600 dalton. This activity coelutes from the chromatographic columns with the peak of a hypoxanthine-like molecule[a].

The enhancer activity is not due to hypoxanthine itself because (i) the synthetic purine derivative is inactive in the employed bioassays and (ii) the biological activity still remains after treatment of the LeF preparation with xanthine oxidase and it is not attributable to the new UV-absorbing peak detected in RP-HPLC. Consequently, we assume that LeF is linked to a compound that co-elutes with the hypoxanthine in our chromatographic system. LeF was purified only 258-fold (Table II) with a recovery of 4%, probably as a possible denaturation of the active molecule during the purification steps. Moreover, the loss of biological activity could also be due to the chromatographic separation of compounds that act synergistically in the crude material.

The biological activity of the LeF does not correspond to that attributed by Sihna *et al.*[7] to the peptides Tyr–Gly and Tyr–Gly–Gly, isolated from a fraction of DLE. In fact, the di- and tripeptides (which are identical with the amino terminal ends of enkephalins) are eluted later than the enhancer activity in the same chromatographic system[b]. Therefore, DLE contains enhancer compound(s) not yet com-

[a] The identification was made by comparison of the UV spectra, from the HPLC behaviours of the natural and synthetic samples and by their amino acid analyses after total acid hydrolysis.

[b] Analysis of *in vitro* PHA-induced transformation by Tyr–Gly, Tyr–Gly–Gly and the DLE shows that the two peptides are not responsible for all the enhancer activity of DLE. In fact, whereas DLE is able to increase the lymphocyte response by about 70%, the two peptides never increase it by more than 20%, at concentrations equivalent to those used for DLE.

pletely identified, acting on cells different from those on which the two enkephalin fragments exert their activity.

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