Journal of Chromatography, 326 (1985) 129–136
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 606

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF DISTINCT EPIDERMAL CELL-DERIVED CYTOKINES

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SUMMARY

High-performance liquid chromatography (HPLC) is useful for the purification and separation of immunoregulatory cytokines, such as macrophage-derived interleukin 1 (IL 1). In addition to macrophages, epidermal cells also release a mediator, epidermal cell (EC) derived thymocyte-activating factor (ETAF), which cannot be separated from IL 1. Moreover, it has been shown recently that EC produce a distinct interleukin 3-like mast cell-activating factor (EC IL 3). This study was performed to investigate whether HPLC may be useful for the separation of EC-derived cytokines, such as ETAF and EC IL 3. For factor production, a murine EC line (Pam 212) was used. ETAF activity was measured using the thymocyte costimulator assay. EC IL 3 was was determined by induction of the proliferative activity of an IL 3-dependent cell line (32 DCL). Using a TSK 125 size-exclusion column and phosphate-buffered saline (pH 7.2) as the mobile phase, ETAF was eluted with an apparent molecular weight of 17 kD, and EC IL 3 with a molecular weight of 28 kD. When EC supernatants were chromatofocused on a Mono P column, ETAF activity was eluted with apparent p*I* values of 6.8, 6.2 and 5.3, and EC IL 3 activity with p*I* 7.8, 7.4 and 7.1. When reversed-phase HPLC (RP-HPLC) (equilibration with water and a 0–100% concave acetonitrile gradient) was applied ETAF exhibited four distinct peaks, whereas EC IL 3 was eluted as one major peak between 70 and 80% acetonitrile. Separation on a Bio-Gel HPHT column with a sodium phosphate gradient was not satisfactory, but the recovery was high. It is concluded that chromatofocusing on Mono P and RP-HPLC are suitable methods for the separation of cytokines, such as ETAF and EC IL 3, both of which are produced by EC.

INTRODUCTION

Keratinocytes, which comprise the majority of epidermal cells (EC) (95%), have recently been shown to release distinct immunoregulatory cytokines[1–5]. For example, epidermal cell-derived thymocyte-activating factor (ETAF) is comparable to macrophage-derived interleukin 1 (IL 1), a mediator with a variety of inflammatory and immunological activities, as recently reviewed[6]. Moreover, EC produce an interleukin 3 (IL 3)-like mast cell-activating factor[2], interferon and epidermal cell-de-

0021-9673/85/$03.30

rived natural killer cell-activating factor[3]. The separation of these biological activities is difficult, because EC-derived cytokines are released only in very small amounts and the methods used are time consuming, involving procedures that may denature the proteins being purified. Therefore, we investigated the utility of high-performance liquid chromatography (HPLC), using mild conditions in order to improve the speed, recovery and the separation of EC-derived cytokines, such as ETAF and IL 3.

EXPERIMENTAL

Cytokine production and bioassays

Supernatants were prepared by incubating the murine keratinocyte cell line Pam 212 in serum-free medium at a density of $1 \cdot 10^6$ cells/ml in the presence of 50 μg/ml silica (Sigma, St. Louis, MO, U.S.A.) for 96 h, as described previously[7].

The enhancement of mitogen-stimulated mouse thymocyte proliferation was used for the detection of ETAF activity, as described elsewhere[7] (C3H/He mice were obtained from the Institut für Versuchtstierzucht, Himberg, Austrias). EC IL 3 activity was measured with the aid of an IL 3-dependent cell line (32 DCL)[2]. Crude preparations usually contained 100 U/ml ETAF activity and 2 U/ml EC IL 3 activity. In order to obtain a reliable index of ETAF and EC IL 3 activity, each assay was compared with a standard ETAF or IL 3 preparation at vasrious dilutions, revealing units of ETAF and IL 3 activity[7].

Concentration of supernatants

The Pam 212 cell supernatants were concentrated 25-fold by lyophilization. They were desalted with the aid of Bio-Gel P-6 DG (Bio-Rad Labs., Richmond, CA, U.S.A.). Eluents were monitored by absorption at 254 nm and the protein-containing fractions were subsequently freeze-dried.

High-performance liquid chromatography

HPLC procedures were carried out at room temperature with two LKB 2150 HPLC pumps (LKB, Bromma, Sweden), an LKB 2152 HPLC controller and a Rheodyne 7125 injector (Rheodyne, Berkley, CA, U.S.A.). Eluents were monitored on a Uvicon 720 LC variable-wavelength detector (Kontron, Vösendorf, Austria) or an LKB 2140 rapid spectral detector. Results were plotted with a Shimadzu C-R1B integrator (Kontron). Fractions were collected with a Frac 100 fraction collector (Pharmacia, Uppsala, Sweden).

The columns used were as follows: a size-exclusion column [Bio-Sil TSK 125, 300 × 7.5 mm I.D. (Bio-Rad Labs.) and an Ultra Pac TSK-GSWP pre-column, 75 × 7.5 mm I.D.; TP 5000 (LKB)] and a Mono P HR5/20 pre-packed, 200 × 5 mm I.D. (Pharmacia). Furthermore, separations were carried out on a Hi-Pore RP-304 reversed-phase (RP) column (250 × 4.6 mm I.D.) with an attached Micro-Guard RP-300 cartridge (Bio-Rad Labs.) and a Bio-Gel HPHT hydroxylapatite (100 × 7.8 mm I.D.) column, protected by a guard column (50 × 4 mm I.D.) filled with a spherical hydrophilic polymer matrix (Bio-Rad Labs.). The flow-rate was 1 ml/min for TSK gels and for the Mono P, 1.5 ml/min for the RP 304 and 0.8 ml/min for the HPHT column.

When the TSK 125 column was used, elution was carried out isocratically with

phosphate-buffered saline (PBS) (pH 7.2). The column effluent was monitored by the absorbance at 210 nm.

In HPLC using hydroxylapatite, the lyophilized sample was dissolved in the starting buffer, containing 0.01 *M* monosodium phosphate and disodium phosphate (pH 7.0), and 0.3 m*M* $CaCl_2$ was added to prevent dissolution of the column matrix. The column was washed extensively with the starting buffer before 100 μl of sample were applied. The columns were eluted by a phosphate gradient, ranging from 0.01 *M* (starting buffer) to 0.5 *M* phosphate, containing 0.01 m*M* $CaCl_2$. The gradient applied was linear from 5 to 17 min, isocratic from 17 to 21 min and linear again from 21 to 30 min.

For chromatofocusing, desalted and lyophilized samples (200 μl) were redissolved in the starting buffer, containing 25 m*M* imidazole–HCl (pH 7.4), and applied to the Mono P column, equilibrated with the starting buffer. Subsequently, the IL 3-containing fractions eluted at pH 7.4 were pooled, concentrated and redissolved in 200 μl of starting buffer, consisting of 25 m*M* glycine in distilled water, adjusting the pH to 10.5 with 1 *N* NaOH. Ampholyte buffer contained 2 ml of Servalyt 3-10 (Serva,. Heidelberg, F.R.G.) in 90 ml of water (pH 6.8). the column was first equilibrated with starting buffer. After applying the sample and after washing the column for 4 min with the starting buffer, elution was carried out with the ampholyte buffer.

Hydrophobic chromatography was performed on an RP 304 column (Bio-Rad Labs.), equilibrated with water. Samples for RP-HPLC were desalted as described above and dissolved in 1 ml water, clarified by centrifugation if necessary, and applied to the column. The water used in HPLC was deionized and distilled in a quartz still. Acetonitrile was obtained from Fluka (Buchs, Switzerland) and was used without further treatment. The column was subsequently eluted with a concave acetonitrile gradient from 0 to 100%. Further gradient conditions were as indicated in the figure legends. Eluted protein was detected by a rapid spectral detector (LKB) between 190 and 360 nm. Fractions of 1 ml were collected, lyophilized, redissolved in water and tested at various dilutions.

RESULTS

Molecular weights (MW) of ETAF and EC IL 3 were determined by HPLC gel filtration on TSK 125 that has been shown previously to be suitable for determining the MW of IL 1/ETAF[8,9]. ETAF activity was eluted as a major peak at approximately 17 kD, whereas most of the EC IL 3 activity had an apparent MW of 28 kD. Although both ETAF and EC IL 3 were separated from the major protein contaminants, considerable overlap between ETAF and EC IL 3 activities precluded their proper separation (Fig. 1). TSK gel filtration yielded 81% ETAF and 87% EC IL 3 activity (Table I).

In HPHT HPLC, ETAF was eluted in two peaks, at 100 and 200–250 m*M* sodium phosphate. EC IL 3 exhibited only one peak at 225 m*M* sodium phosphate. EC IL 3 was therefore eluted with some of the ETAF activity, indicating that HPHT is not suitable for distinguishing between ETAF and EC IL 3 (Fig. 2). As with TSK, HPHT yielded 86% ETAF and 83% EC IL 3 activity (Table I).

In chromatofocusing with a pH gradient from pH 7.4 to 4, ETAF exhibited three isoelectric points (IEP) of p*I* 6.8, 6.2 and 5.3. In contrast, EC IL 3 exhibited

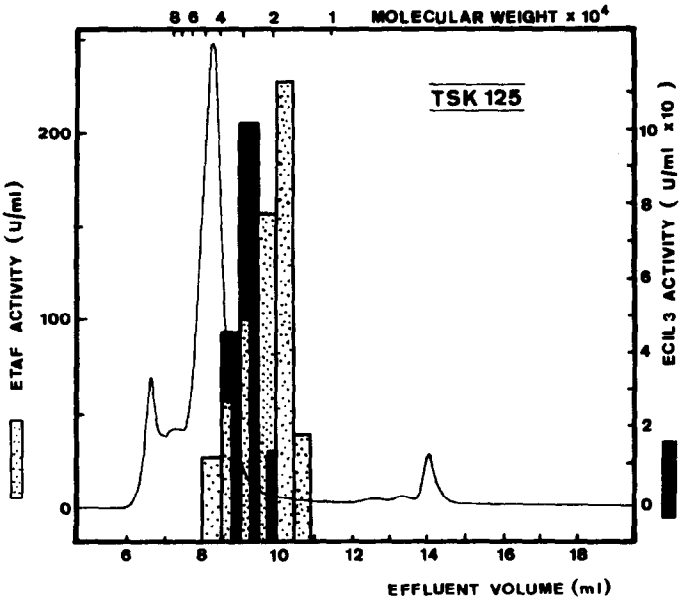

Fig. 1. Bio-Sil TSK 125 size-exclusion column. Elution was carried out with PBS, at a flow-rate of 1 ml/min. ETAF activity (dotted areas) was eluted within an MW range of 15–17 kD, whereas the EC IL 3 activity (black areas) exhibited a MW of approximately 28 kD.

two distinct IEP of p*I* 7.4 and 7.1. Because some EC IL 3 was eluted within the plateau of the pH gradient, these fractions were pooled and rechromatographed on Mono P. When a pH gradient, ranging from pH 10 to 7.0, was used, EC IL 3 showed two peaks of activity at pH 7.8 and 7.4. EC IL 3 therefore has three isoelectric points at p*I* 7.8, 7.4 and 7.1. Moreover, isoelectric focusing on Mono P is suitable for the

TABLE I

PURIFICATION OF EC IL 3 AND ETAF

Column		*EC IL 3*			*ETAF*		
		ml	*U/ml*	*Total U*	*ml*	*U/ml*	*Total U*
TSK 125	Applied	0.2	1500	300	0.2	30 100	6020
	Eluted	1.5	173.3	260	3.0	1633	4900
	Recovery			86.6%			81.4%
HPHT	Applied	0.2	1500	300	0.2	6200	1204
	Eluted	6.0	41.8	251	14.0	73.9	1034
	Recovery			83.7%			85.9%
Mono P	Applied	0.2	1500	300	0.2	31 100	6020
	Eluted	10.0	26.0	260	9.0	544.4	4900
	Recovery			86%			81.4%
RP 304	Applied	0.2	935	187	0.2	6.02	1204
	Eluted	11.25	12.9	145.1	20.0	64.0	1280
	Recovery			77.5%			106%

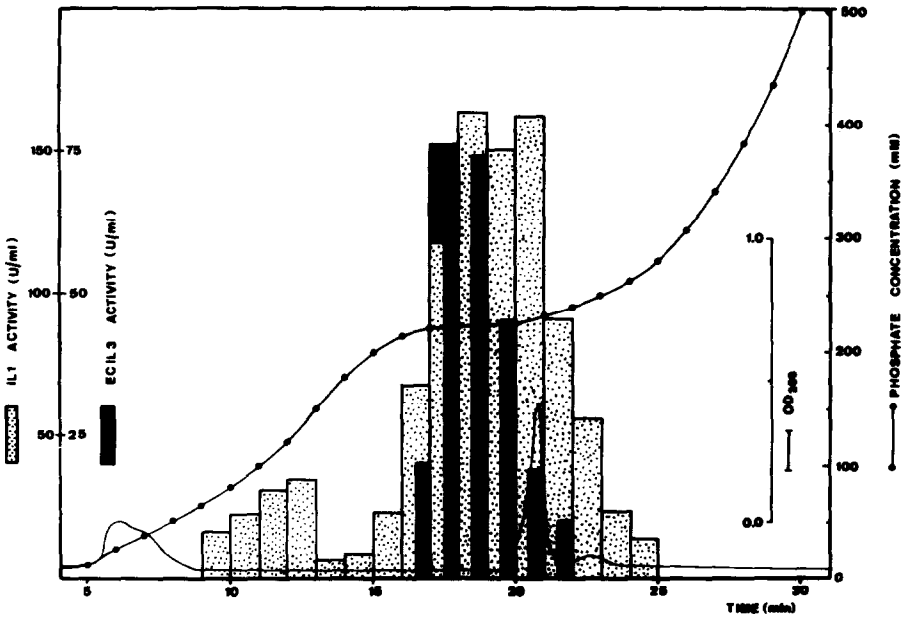

Fig. 2. Bio-Gel HPHT hydroxylapatite chromatography of EC cytokines. Applying a phosphate gradient at a flow-rate of 0.8 ml/min (●–●). ETAF activity (dotted areas) was eluted in two peaks at 100 and 200–250 m*M* sodium phosphate. EC IL 3 activity (black areas) was eluted in only one peak at 225 m*M* sodium phosphate.

separation of ETAF and EC IL 3 activity (Fig. 3). Although ETAF and EC IL 3 were eluted with three different peaks of activity, the recoveries were 81 and 86%, respectively (Table I).

RP-HPLC was also tested for the purification of EC-derived mediators. When an acetonitrile gradient was applied ETAF was eluted in several peaks, whereas EC IL 3 exhibited only one peak (Fig. 4). Therefore, RP-HPLC has proved to be useful for the purification and separation of EC IL 3. After RP-HPLC, 77.5% of EC IL 3 and 106% of ETAF were recovered (Table I).

DISCUSSION

Cytokines derived from both lymphocytes and epidermal cells have recently been purified by HPLC procedures[8,9]. As HPLC is less time consuming than conventional chromatographic procedures and usually provides a good recovery of proteins, different columns were tested for their ability to separate cytokines present in EC supernatants. The difference in molecular weight did not result in a clear separation on TSK 125 of ETAF from EC IL 3. The MW of ETAF is close to values found previously by other techniques and is similar to that of macrophage-derived IL 1[8]. However, cleavage products of ETAF and IL 1 have been observed[10,11], and activities recovered in the high-molecular-weight range may represent aggregates or association products with carrier proteins. The MW of EC IL 3 determined by TSK 125 HPLC is also in agreement with values previously obtained by conventional chromatography and by SDS–PAGE of lymphocyte-derived IL 3[12].

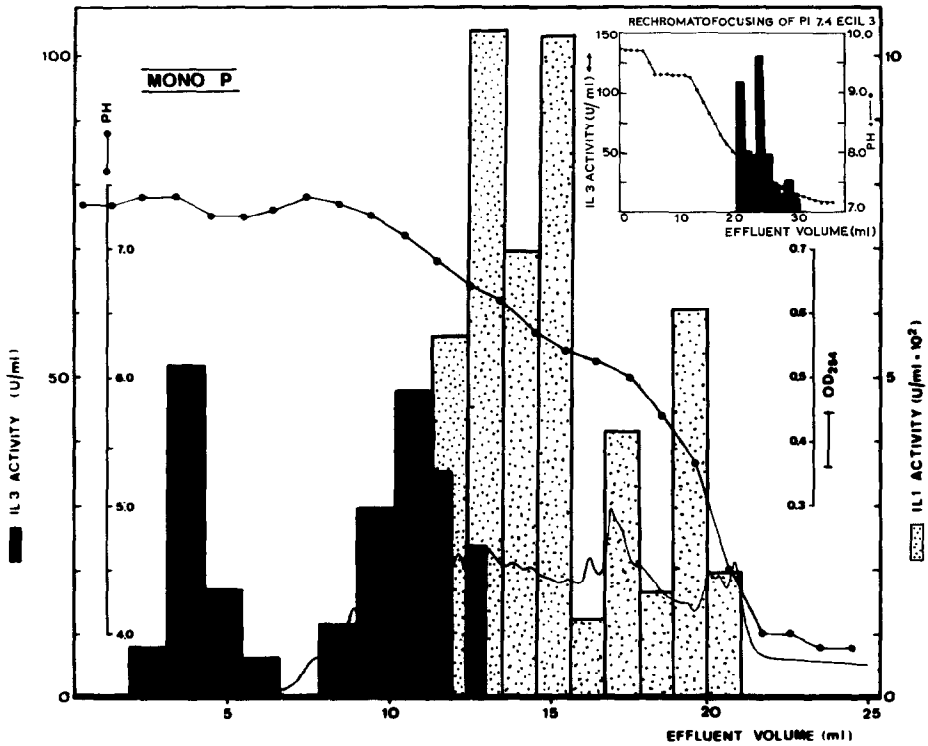

Fig. 3. Mono P chromatofocusing of $PAM_{212}SN$. Applying an internal pH gradient at a flow-rate of 1 ml/min (●—●), ETAF activity (dotted areas) was eluted at pH 6.8, 6.2 and 4.3; EC IL 3 (black areas) was eluted at pH 7.8, 7.4 and 7.1.

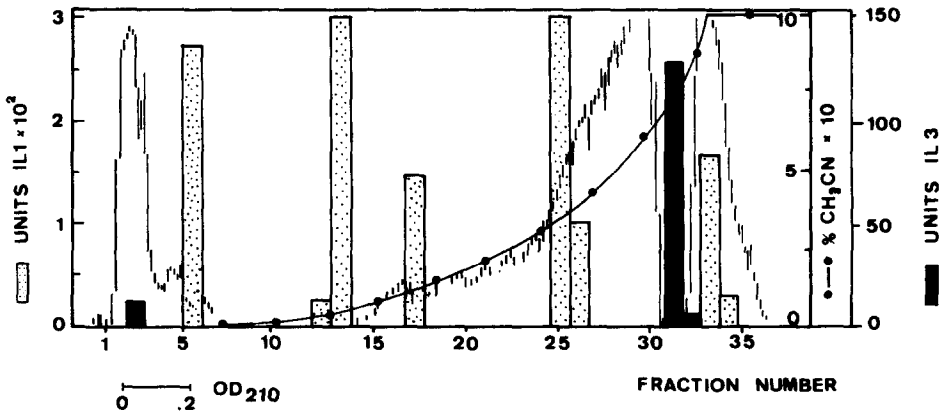

Fig. 4. RP 304 reversed-phase chromatography of EC cytokines. With an acetonitrile gradient (●—●), ranging from 0 to 100%, at a flow-rate of 1.5 ml/min, ETAF activity (dotted areas) was eluted as five distinct peaks, whereas EC IL 3 activity (black areas) showed one sharp peak at 80% acetonitrile.

The Bio-Gel HPHT column separates proteins by surface binding to phosphate binding sites. Because of the high protein binding capacity and high resolution, this method appeared useful for the purification of both IL 1 and ETAF[8]. However, on HPHT only one species of ETAF was separated from EC IL 3, whereas another species was eluted with EC IL 3, indicating that HPHT appears to be unsuitable for the separation of these mediators. However, both HPHT and TSK 125 chromatography may be used to separate EC cytokines from major protein contaminants. The unusually high yield of biological activity is in accordance with previous findings and can be explained by the separation of an inhibitor present in crude EC supernatants[5].

The isoelectric points of ETAF and EC IL 3 were determined on Mono P. Like lymphocyte IL 3, EC IL 3 had three IEP between p*I* 7.8 and 7.1. ETAF exhibited three IEP between pH 6.8 and 5.3, whereas previous data revealed only one IEP at pH 4.8 for murine ETAF[5]. The present findings were also confirmed when EC supernatants were chromatofocused on TSK IEX 540 DEAE (unpublished observation). It therefore remains undetermined whether HPLC can separate these biologically active components from a single precursor molecule. However, as the IEP values of ETAF and EC IL 3 are within distinct ranges of the pH gradient and the recovery is good, chromatofocusing on Mono P may be used for separation of these EC-derived mediators.

RP chromatography has recently been introduced for the purification and separation of cytokines, such as the separation on RP 304 of human EC-derived killer cell-augmenting factor, interferon, ETAF[3]. In the absence of trifluoracetic acid, murine ETAF showed several distinct peaks at different acetonitrile concentrations, whereas EC IL 3 was eluted as a single peak. Both activities were recovered in very high yield.

In conclusion, all HPLC procedures applied provided good recoveries of biological activity, were highly reproducible and separated EC cytokines from protein contaminants. For separation of ETAF from EC IL 3, chromatography on Mono P and RP 304 proved most effective. Moreover, the speed of HPLC separations makes it possible to purify larger amounts of EC cytokines, such as ETAF and EC IL 3, for further biochemical characterization and sequence analysis. It also should be possible to investigate the different biological properties of these mediators in detail and differentiate them from mononuclear cell-derived factors, including IL 1 and IL 3.

ACKNOWLEDGEMENTS

We thank Dr. J. Graf for critically reviewing the manuscript and Mrs. M. Bednar for excellent secretarial assistance. This work was supported by the Medizinisch Wissenschaftlicher Fonds des Bürgermeisters der Stadt Wien and the Jubiläumsfonds der Österreichischen Nationalbank P 2082.

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