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High-performance capillary electrophoretic analysis of inflammatory citokines in human biopsies

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Abstract

The detection of inflammatory citokines in pathological tissue samples is important as an indicator of disease severity and persistence. Capillary zone electrophoresis (CZE) has been shown to be a good technique for the detection and measurement of tissue-bound citokines in frozen biopsy specimens. CZE separations in polyethylene glycol-coated capillaries, performed at neutral pH and sub-ambient temperatures was found to give the most consistent and reliable results.

1. Introduction

The measurement of low-molecular-mass peptides (citokines) associated with immunological regulation and the inflammatory process has gained immense popularity during recent times [1,2]. Measurement of circulating levels of interleukin-1 (IL-1), interleukin-6 (IL-6), γ-interferon (γIFN) and tumour necrosis factor α (TNFα) (inflammatory citokines) can act as sensitive indicators of ongoing inflammation, while levels of interleukin-2, -3, -4 and -5 may indicate an active immune response, especially those involving lymphocyte activation [2]. Recently, a new family of citokines have become of interest mainly due to their ability to attract host cells into inflammatory sites [3–5]. This family, the chemokines, consists of IL-8, monocyte chemotactic protein-1 (MCP-1) and Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES) as well as several other chemoattractive peptides. Measurement of this latter family may have pathobiological significance as chemokines have been shown to attract T-cells, neutrophils and eosinophils to sites of immune activity [3–5].

We have been engaged for some time in examining the pathobiological events which take place in human immunodeficiency virus (HIV)-associated renal disease and have described several features which are characteristic of this nephropathy, such as anti-idiotypic antibody responses [6] and circulating immune complexes leading to renal deposition and glomerular inflammation [7]. Histopathological examination of biopsy materials from patients with HIV-associated nephropathy often demonstrate the presence of an inflammatory cell infiltrate in the renal interstitial tissue in addition to inflammatory glomerular disease [7]. This evidence, along with the finding that the citokine, transforming growth factor β, can increase the expression of

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HIV gene in transfected mesangial cells [8], led us to believe that both the inflammatory citokines and the chemokines play an important role in the pathogenesis of HIV-associated renal disease.

Although several sensitive techniques have been developed to study circulating levels of inflammatory citokines [9–12], correlations between plasma concentrations and inflammation severity have been hard to make. One answer is that citokines can act as simulators of both their own and other citokine production, thus causing a cascade effect [1,2]. In such a scenario, measurement of plasma concentrations becomes questionable and microchemical techniques, capable of measuring tissue concentrations of citokines are beginning to be developed.

Since its introduction, high-performance capillary electrophoresis (HPCE) has become a popular analytical tool in protein chemistry [13–17], mainly due to its ability to provide rapid analysis of extremely small samples, high resolution, and computer-based quantification [18]. HPCE has been successfully applied to the detection of citokines in pharmaceutical preparations [19] and homogenates of human tissue sections [20]. In an attempt to study the pathobiology of renal disease in AIDS patients, we have developed a technique for measuring inflammatory citokines in frozen sections of renal biopsy material, using a combination of microdissection and HPCE.

2. Experimental

2.1. Reagents

Recombinant human citokines, IL-1β, IL-6, γIFN and TNFα were obtained from Genzyme (Cambridge, MA, USA). The human chemokines, IL-8, RANTES and MCP-1 were obtained from R & D Sciences (Minneapolis, MN, USA). Antibodies directed against each citokine were obtained from R & D Sciences and labelled with alkaline phosphatase (Sigma, St. Louis, MO, USA) by standard techniques. Polyethylene glycol diglycidyl ether (M_r 600) and γ-glycidoxy-propyltrimethoxysilane was purchased from Polysciences (Warrington, PA, USA). HPCE buffers were obtained from ISCO (Lincoln, NE, USA) and all other chemicals were purchased from Aldrich (Milwaukee, WI, USA). All solutions were passed through 0.2-μm nitrocellulose filters (Millipore, Bedford, MA, USA) prior to use.

2.2. Instrumentation

All separations were performed on a ISCO 3140 capillary electrophoresis system using the integrated method and analysis program supplied by the manufacturer (ISCO). Unmodified internal surface, fused-silica capillaries (375 μm O.D. $\times$ 75 μm I.D.) were obtained from ISCO and cut to a standard length of 75 cm, 60 cm to the detector cell. HPCE separations were performed at a controlled capillary chamber temperature of 15°C. Integrations of the isolated peaks were analyzed (peak area) by the data management program provided with the instrument and they were compared to the peak areas of recombinant citokines, run under identical conditions. Chromatograms were stored as ASCII files and plotted using Inplot 4.03 (GraphPad Software, San Diego, CA, USA) and a Hewlett-Packard Deskjet printer.

2.3. Capillary coating

The interior surfaces of the unmodified capillaries were coated with polyethylene glycol polyether according to the technique described by Nashabeh and El Rassi [21]. Following installation in the instrument, the capillary was purged five times with 100 mM phosphate buffer and filled with fresh buffer prior to use.

2.4. Microdissection of patient biopsy sections

Cryostat sections (10 μm) were placed on cold (-5°C) Mylar coverslips, briefly stained in 0.01% aqueous cotton blue (to aid morphological identification of the tissue), and placed into a pre-cooled micro-incubation chamber (Narishige, Greenvale, NY, USA). Defined morphological areas (glomeruli or tubular inter-

stitium) were microdissected from the surrounding tissue with a M-155 glass needle micromanipulator (Narishige). Following microdissection, the areas of interest were flooded with 25 μl of warm (22°C) buffer (100 m*M* phosphate buffer, pH 7.0, containing 0.2% Nonidet 40), administered with a M-6 micro-injection system (Narishige). Recovery of the injected fluid was performed with the same instrument, clarified by centrifugal ultrafiltration through either an M_r 30 000 or an M_r 10 000 filter (10 000 *g* for 10 min in a Beckman Airfuge: Beckman Instruments, Palo Alto, CA, USA), and the filtrate analyzed by HPCE. Prior to HPCE analysis, the total protein concentration of each sample was measured by direct spectrophotometry at 280 and 260 nm and normalized to 10 μg protein/ml [20].

2.5. HPCE analysis of microdissected samples

Approximately 20-nl samples were introduced into either uncoated or polyethylene glycol-coated capillaries, filled with 100 m*M* phosphate buffer, by vacuum injection and electrophoretically separated at 27 kV constant voltage. The migration of the sample components were monitored by on-line UV detection at 200 nm and the electropherogram directly read into a computerized recording system. Continuous fractions were collected on a linear modification of the circular membrane-based system described by Cheng *et al.* [22], adapted from the original description of the instrument developed by Eriksson *et al.* [23], using a polyvinylidene difluoride (PVDF; Immobilon-P membrane, Millipore) membrane as the collection device.

2.6. Fraction collection of HPCE separated peaks

The membrane-based fraction collector consisted of a rectangle of Perspex block onto which a stainless-steel plate was attached by four screws. This plate formed the ground electrode of the system and was attached to the cathodal end of the electrophoresis apparatus. The steel plate was covered with three sheets of Whatman 3MM filter paper which previously been soaked in running buffer. Finally, the PVDF membrane was pre-wetted in methanol before being permeated with running buffer and carefully laid on top of the uppermost filter paper, The entire assembly was secured into place with a plastic clip, placed at either end of the longitudinal axis of the instrument. The outlet of the capillary was lowered until it made contact with the membrane and the electrophoretic separation performed as described above. An attached electrical motor was used to advance the fraction collector during the electrophoresis run and continuous fractions were collected and quantitatively measured using a chemiluminescence-enhanced immunoassay.

The efficiency and reliability of the membrane fraction collector was checked by collecting individual peaks during the electrophoretic separation. Separated peaks were collected by recording the migration time of each peak and then calculating its elution time (t_e) from the following formula [20]:

$$t_e = (L/L_d)t_d$$

where L is the total length of the capillary, L_d is the capillary length to the detector and t_d is the time when the peak passes the detector.

Fractions were collected by interrupting the current just before the peak elution time and placing the detector end of the capillary and the electrode into a 75-μl vial containing 10 μl of running buffer, placed under 25 μl of mineral oil. The current was reapplied and the peak forced to electromigrate into the collection vial. This process was repeated for each peak.

2.7. Chemiluminescence-enhanced immunoassay of HPCE isolated citokines

Citokine concentrations in fractions collected from each HPCE separation were measured by chemiluminescence-enhanced enzyme-linked immunoassay (CHEM-ELISA) [20,24], using specific alkaline phosphatase-labeled antibodies, directed against each cytokine. Briefly, the membrane was removed from the fraction collector and the central portion (along the capillary delivery line) divided into seven strips. Each

strip was incubated with a specific enzyme-labelled antibody, directed against one of the citokines or chemokines of interest, for 24 h at room temperature. The strips were washed in 100 m*M* phosphate buffer, pH 7.2 and the bound antibodies detected following their reaction with AMPPD, an alkaline phosphatase chemiluminescence substrate (Tropix, Bedford, MA, USA). The CHEM-ELISA results were analyzed by the ANELISA-R software package (Man-Tech Associates, Buffalo, NY, USA).

3. Results and discussion

Development of ideal conditions for the isolation and measurement of citokines centered around the ability of HPCE to separate a mixture of seven pure, recombinant citokines, using capillary zone electrophoresis (CZE) in both uncoated and coated capillaries. Electrophoresis in uncoated capillaries was able to separate seven of the eight citokines, although in less than optimal amounts, especially γIFN and IL-8. At the 10 pg/ml level the uncoated capillary produced results indicating a approximate 80% recovery for all citokines except γIFN and IL-8, which were recovered at 71 and 66%, respectively. This poor recovery was thought to be due to retardation and adsorption of the different molecules to charged groups on the uncoated capillary walls: an effect which has been reported to give rise to band broadening and low solute recovery rates by other investigators [21,25,26]. Protein and peptide adsorption to capillary walls is a common problem associated with HPCE and several workers have reported techniques for overcoming this situation. Manipulations such as, changing the pH of the running buffer [21,27,28], addition of salts [29,30], and chemical modification of the inner capillary surface have all been suggested [21,31–35]. In our experience, coating the internal capillary surfaces with polyethylene glycol greatly enhanced both the resolution of the electropherogram as well as improving the isolation and recovery of all seven citokines. At 10 pg/ml, both γIFN and IL-8 were recovered at 93 and 94% with coefficients of variation (C.V.) of 10.8 and 8.5%, respectively. HPCE analysis of the other citokines, using coated capillaries, demonstrated a 95% recovery (except MCP-1 at 93%) with C.V.s ranging from 3.1 to 11.8% (Table 1).

Although HPCE separations generate considerable amounts of heat within the capillary, this factor did not appear to affect the migration times of either the citokines or the chemokines in our system. However, all separations were performed at 15°C, the lowest practical temperature on our instrument, to protect the analytes.

In an electropherogram of a unfiltered, whole extract of a microdissected glomerulus, a total of 40 peaks could be resolved by CZE using polyethylene glycol-coated capillaries, but only three of the seven citokines could be identified. In an attempt to improve the resolution of the electropherogram, the microdissected tissue extract was centrifugally filtered through an M_r 30 000 cut-off membrane filter. Since the citokines of interest were below M_r 30 000 [1–4], it was thought that selective molecular mass filtration would improve the HPCE analysis. As shown in Fig. 1A, pre-filtration greatly improved the resolution of the electropherogram, reducing the number of resolved peaks to 20 and allowing identification of the inflammatory citokines IL-1, IL-6, TNFα, γIFN and the chemokine, RANTES. However, the other two chemokines could still not be resolved under these conditions. In an attempt to improve further the resolution of the electropherogram, we passed the filtrate from the M_r 30 000 filter through an M_r 10 000 cut-off filter and reanalyzed the filtrate. Fig. 1B shows that this second filtration was successful, producing eight peaks and allowing identification of all of the chemokines.

Fig. 2 illustrates our findings on extracts of microdissected renal interstitial tissue, using M_r 30 000 filtration (Fig. 2A) and M_r 10 000 filtration of the M_r 30 000 filtrate (Fig. 2B). Analysis of the M_r 30 000 filtrate demonstrated the presence of 29 peaks but still gave enough resolution in order to identify the four inflammatory citokines and RANTES. Analysis of the M_r 10 000 filtrate demonstrated the presence of 12 peaks, the three largest of which contained the

Table 1
Precision of HPCE technique for the determination of 10 pg/ml citokine and chemokine standards using uncoated and coated capillaries

Cytokine	Amount measured (mean ± S.D., $n = 10$) (pg/ml)	C.V. (%)	Recovery (%)
Uncoated capillary			
IL-1	8.3 ± 1.9	22.9	83
IL-6	8.5 ± 2.2	25.9	85
TNFα	8.0 ± 2.5	31.2	80
γIFN	7.1 ± 3.1	43.7	71
IL-8	6.6 ± 3.4	51.5	66
RANTES	8.2 ± 1.7	20.7	82
MCP-1	7.9 ± 2.6	32.9	79
Coated capillary			
IL-1	9.6 ± 0.3	3.1	96
IL-6	9.4 ± 0.7	7.5	94
TNFα	9.5 ± 0.8	8.4	95
γIFN	9.3 ± 1.0	10.8	93
IL-8	9.4 ± 0.8	8.5	94
RANTES	9.5 ± 0.6	6.3	95
MCP-1	9.3 ± 1.1	11.8	93

analytes RANTES, IL-8 and MCP-1, respectively. Experiments using sample filtration on mixtures of recombinant citokines demonstrated that the loss due to adsorption to the filter was consistently 2–5%.

Using the HPCE technique, to examine sections of renal biopsy tissue following microdissection of either the glomeruli or the interstitial tissue, we have been able to demonstrate the presence of significantly higher levels of inflammatory citokines (IL-1, TNFα and IL-6) in both the glomerulus and the interstitial tissue of patients with HIV-associated glomerulonephritis as compared to non-HIV patients with similar diseases. Glomerular levels of 77 ± 15 pg/μg extracted protein, 83 ± 22 pg/μg extracted protein and 47 ± 15 pg/μg extracted protein could be demonstrated for IL-1, TNFα and IL-6, respectively in the HIV group as compared with 8 ± 2 pg/μg of extracted protein, 14 ± 8 pg/μg extracted protein and 6 ± 2 pg/μg extracted protein for the same citokines in the control group. Interstitial tissue samples showed a similar pattern with levels of 40 ± 12, 47 ± 16 and 29 ± 10 pg/μg extracted protein for IL-1, TNFα and IL-6. Interstitial levels of these citokines in the control group were barely detectable with the highest level being for TNFα at 8.2 ± 2 pg/μg extracted protein.

In addition, we have been able to show marked elevations of the chemokines in both glomerular and interstitial tissue obtained from HIV-associated glomerulonephritis, with significantly higher levels of all three chemokines in the interstitial tissue. Glomerular levels of 16.4 ± 1.4 for IL-8, 11.5 ± 1.4 for RANTES and 11.3 ± 1.3 pg/μg extracted protein for MCP-1 could be demonstrated in the HIV groups as compared to 6.9 ± 1 pg/μg extracted protein for IL-8 and undetectable levels of RANTES and MCP-1 in the control group. Of greatest interest were the levels found in the interstitial tissue extracts from both the HIV and control groups. Chemokine levels of 28.2 ± 5 for IL-8, 16.1 ± 3.5 for RANTES and 19.0 ± 4 pg/μg extracted protein for MCP-1 could be shown in the HIV samples as compared to 6.3 ± 1.5, 2.4 ± 1 and 2.6 ± 1 pg/μg extracted protein for the same chemokines in the control group (Table 2). Since interstitial cellular infiltrates are known to be part of the pathology

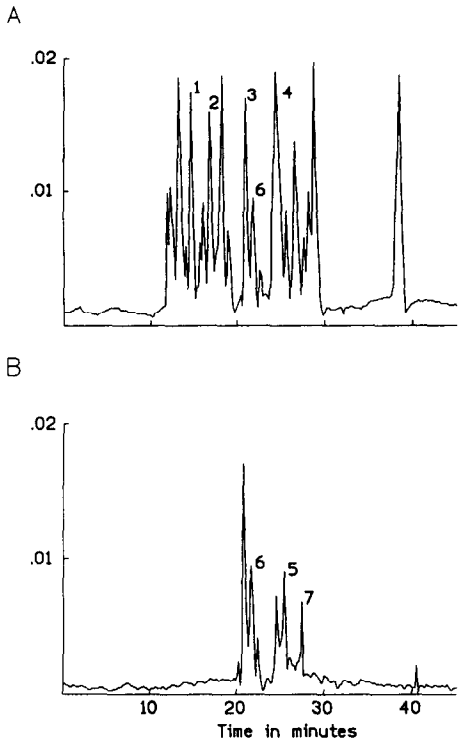

Fig. 1. HPCE profiles of microdissected renal glomerular tissue extracts following filtration through (A) an M_r 30 000 cut-off membrane filter and (B) an M_r 10 000 cut-off membrane filter. Separation performed in a 75 cm (60 cm to detector) × 75 μm I.D. polyimide-coated capillary at 15°C. Voltage: 27 kV; buffer: 0.1 *M* phosphate, pH 7.0; detection at 200 nm, 0.02 AU. Peaks: 1 = IL-6; 2 = γIFN; 3 = TNFα; 4 = IL-1; 5 = IL-8; 6 = RANTES; 7 = MCP-1.

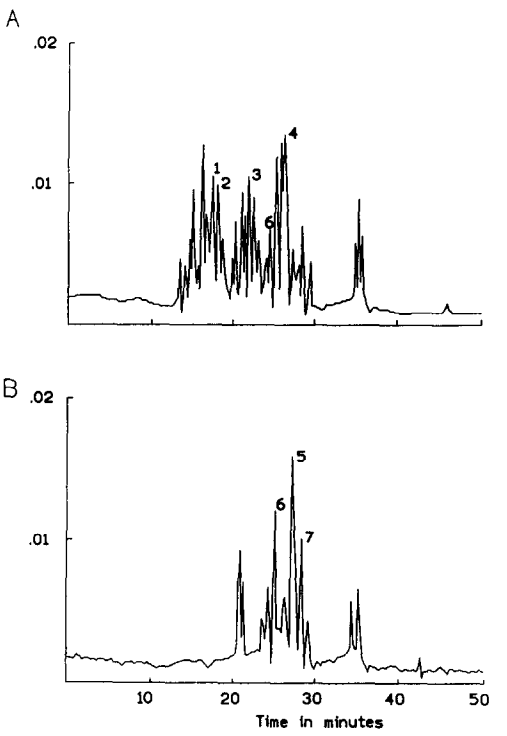

Fig. 2. HPCE profiles of microdissected renal interstitial tissue extracts following filtration through (A) an M_r 30 000 cut-off membrane filter and (B) an M_r 10 000 cut-off membrane filter. Running conditions as described in Fig. 1. Peaks: 1 = IL-6; 2 = γIFN; 3 = TNFα; 4 = IL-1; 5 = IL-8; 6 = RANTES; 7 = MCP-1.

of HIV-associated renal disease [6,7], and the chemokines have been shown to be strong attractants for many immunologically active cells [3–5], these findings were particularly exciting and fitted with the histopathological findings in the HIV group.

Measurement of the isolated citokines by CHEM-ELISA was used to both identify the citokine and chemokine peaks and verify the validity of the HPCE technique. As shown in Table 2 the levels detected by CHEM-ELISA were approximately three-fold greater than those measured by the HPCE system. This increase was found to be due to several factors, including an intensifying factor produced by the chemiluminescence reaction. Further experimentation demonstrated that quantitation of known citokine standards by CHEM-ELISA produced less accurate results than similar results produced by HPCE, especially in the 2–10 pg/ml range. Citokine measurement by CHEM-ELISA also required isolation of the different citokines prior to analysis in order to prevent cross-reactions occurring between the different citokines and the enzyme-labelled anti-citokine antibodies. Although these problems proved that CHEM-ELISA was unsuitable for use as an enhanced post-separation detection technique, it was useful in verifying the identity of the HPCE peaks. Considering the time involved and the ease of operation, we feel that the HPCE technique is sensitive and reliable enough to be used for

Table 2
Concentrations of recovered citokines in renal tissue

Cytokine	Tissue	Detection technique[a]	
		HPCE	CHEM-ELISA
HIV Group			
IL-1	Glomerulus	77 ± 15	241 ± 52
IL-6	Glomerulus	47 ± 15	143 ± 40
TNFα	Glomerulus	83 ± 22	266 ± 35
γIFN	Glomerulus	27 ± 12	90 ± 25
IL-8	Glomerulus	16.4 ± 1.4	51.5 ± 5
RANTES	Glomerulus	11.5 ± 1.4	39.6 ± 6
MCP-1	Glomerulus	11.3 ± 1.3	36.5 ± 5
IL-1	Interstitium	40 ± 12	132 ± 21
IL-6	Interstitium	47 ± 16	150 ± 35
TNFα	Interstitium	29 ± 10	93 ± 15
γIFN	Interstitium	12 ± 5	39 ± 10
IL-8	Interstitium	28.2 ± 3	91 ± 10
RANTES	Interstitium	16.1 ± 3.5	49.6 ± 10
MCP-1	Interstitium	19.0 ± 4	60.8 ± 12
Control group			
IL-1	Glomerulus	8.2 ± 2	27 ± 6.5
IL-6	Glomerulus	6.2 ± 2	21 ± 5
TNFα	Glomerulus	14.8 ± 8	49 ± 16
γIFN	Glomerulus	5 ± 2	15 ± 8
IL-8	Glomerulus	6.9 ± 1	24 ± 5
RANTES	Glomerulus	< 2	< 10
MCP-1	Glomerulus	< 2	< 10
IL-1	Interstitium	2.5 ± 1	< 10
IL-6	Interstitium	< 2	< 10
TNFα	Interstitium	8.2 ± 2	28 ± 8
γIFN	Interstitium	< 2	< 10
IL-8	Interstitium	6.3 ± 1.5	21 ± 5
RANTES	Interstitium	2.4 ± 1	< 10
MCP-1	Interstitium	2.6 ± 1	< 10

[a] Values expressed in pg/μg of extracted protein.

routine screening of tissue citokines in biopsy materials obtained from human subjects.

4. Conclusions

From this early work, we feel that the HPCE technique offers a unique way to study citokines in human biopsy material. In the study briefly outlined above, we have been able to use HPCE generated citokine profiles to study the pathobiology of renal disease in HIV-positive patients, at the tissue level.

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