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Microdialysis–immunoaffinity capillary electrophoresis studies on neuropeptide-induced lymphocyte secretion

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Abstract

A micro-sampling procedure has been developed for studying lymphocyte secretion of biologically important peptides in low cell density cultures. The technique is based on microdialysis recovery of the analytes of interest coupled with immunoaffinity capillary electrophoresis separation of the microdialysis samples and laser-induced fluorescence detection. Although the technique is able to recover secreted materials only at the 5–10 cell level, the detection system has a limit of detection (LOD) in the attomole (10^{-18} *M*) range. This degree of sensitivity indicates that the system has the potential to measure secreted products at the single cell level. An added advantage of this system over other sampling techniques is that the microdialysis probe allows continuous sampling over time. ©1997 Elsevier Science B.V.

Keywords: Lymphocyte secretion; Peptides; Neuropeptides

1. Introduction

Interest in neurological regulation of host defense mechanisms has greatly increased over the past few years mainly due to the increasing number of reports demonstrating the presence of neuropeptide receptors on immune cells [1–4]. Additionally, there is evidence linking the presence of neuropeptides with a number of immune-mediated clinical events. The presence of circulating neuropeptides, especially substance P (SP), has been associated with a number of disease states such as inflammatory joint disease [5], asthma-induced bronchial inflammation [6], allergic nasal mucosal inflammation [7], experimental perivascular inflammation [8] and UV-irradiation-induced cutaneous inflammation [9], However, few studies have attempted to examine the influence of SP on immune cells at the single cell level mainly due to the lack of suitable techniques for performing such studies.

Microdialysis is a technique whereby a small probe equipped with a short length of dialysis membrane at its tip is implanted into the tissue or organ of a living animal. The infusion of an isotonic solution using a precisely controlled flow-rate allows molecules to be selectively removed from the extracellular matrix by establishing a steady-state osmotic flux across the dialysis membrane. The advantage of this type of sampling technique is that the recovered materials are relatively clean and often do not require extensive preparation prior to analysis [10]. Microdialysis sampling has been applied to the continuous monitoring of a number of important drugs and metabolites in-vivo [11–13] in both living animals

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and human subjects and can readily be adapted for in-vitro cell culture use.

In the present studies, we have utilized the microdialysis technique to examine secretory events (cytokine release) which take place following SP stimulation of cultured T-lymphocytes. Furthermore, we have developed a two-dimensional immunoaffinity capillary electrophoresis (ICE) system in order to detect and measure the cytokines recovered by micro-dialysis. ICE is based on separation via immobilized antibody specificity in the first dimension followed by separation and detection of the immunoreactive analytes by zone electrophoresis coupled with laser-induced fluorescence (LIF) detection in the second dimension.

2. Experimental

2.1. Reagents

SP was obtained from Bachem Biosciences (King of Prussia, PA, USA) and dissolved in 0.01 *M* phosphate buffered saline (PBS), pH 7.2, to a final concentration of 1 μg/ml. Recombinant cytokines (interleukin (IL)-2, -4, -5, -10, -12) and their corresponding anticytokine antibodies were obtained from R and D Systems (Minneapolis, MN, USA) and reconstituted to stock solutions of 1 μg/ml in PBS. Sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SSMCC), the ImmunoPure $F(Ab')_2$ preparation kit and Clelands reagent were purchased from Pierce (Rockford, IL, USA). Fused-silica capillaries (100 μm I.D.) with unmodified internal surfaces were obtained from Phenomenex (Torrance, CA, USA) and cut to a length of 30 cm (20 cm to the detector cell) prior to antibody coating. 3-Aminopropyltriethoxysilane was purchased from Polysciences (Warrington, PA, USA) and all other chemicals were purchased from either Aldrich (Milwaukee, WI, USA) or Sigma (St. Louis, MO, USA). Prior to use all solutions were passed through 0.2-μm nitrocellulose filters (Millipore, Bedford, MA, USA).

2.2. Preparation of the immunoaffinity capillary

Divalent $F(Ab')_2$ fragments were prepared from each of the anti-cytokine antibodies using the Pierce ImmunoPure $F(Ab')_2$ preparation kit according to the manufacturers instructions. The fragments were further reduced to monovalent FAb fragments by incubation with equal volumes of 200 m*M* Clelands reagent for 30 min at 37°C [14]. Following this digestion, equal volumes of each anti-cytokine FAb were mixed prior to coating the capillary.

The interior surfaces of unmodified fused-silica capillaries were silanized prior to the covalent attachment of functional antibody fragments as previously described [15]. One third of each capillary was filled with 10% aqueous 3-aminopropyltriethoxysilane by capillary action and incubated at 100°C for 60 min. This treatment was repeated four times before filling the capillaries with 10 m*M* HCl and incubating at 100°C for 60 min. The capillaries were washed once in distilled water before preparing the maleimide-activated surface. This was achieved by filling one third of the capillary with a solution containing 1 mg/ml SSMCC dissolved in 50 m*M* sodium borate, pH 7.6, and reincubating at 30°C for 60 min. Following this incubation, the capillary was flushed with 50 m*M* borate, filled with the FAb mixture and incubated overnight at 4°C. Finally, the capillaries were flushed three times with 100 m*M* phosphate buffer, pH 7.4, and stored in this buffer. Following installation in the capillary electrophoresis instrument, the capillary was purged five times with 100 m*M* phosphate buffer and then filled with fresh buffer prior to use.

2.3. Preparation and stimulation of lymphocyte cultures

Blood was drawn from consenting normal humans (according to a protocol which was approved by the institutional IRB) and centrifuged at 400 *g* for 15 min to separate the plasma from the cells before carefully diluting the blood pellet with 2 ml of RPMI 1640 medium (Sigma). The cell suspension was then washed twice in medium before finally being resuspended in 10 ml of medium, which had been supplemented with 10% fetal calf serum. A 5-ml volume of a 0.5 mg/ml suspension of anti-human CD3 (pan T-lymphocyte) antibody-coated magnetic beads (Dynal, Great Neck, NY, USA) was added to each 10-ml cell suspension. These were then incubated for 10 min at room temperature. The bead-coated cells were isolated by placing a cobalt–

samarium magnet (Dynal) against the side of the tube and decanting the non-attached cell suspension. The attached cells were washed in medium by removing the magnet and suspending the coated cells in 10 ml RPMI 1640. The magnet was replaced and the medium decanted. The wash procedure was repeated three times before finally suspending the cells in 10 ml RPMI-1640 containing 10% serum-free supplement, 2 m*M* L-glutamine, 100 U/ml penicillin and 10 mg/ml streptomycin. The cells were cultured overnight at 37°C to detach the beads [16], washed twice in medium and the cell density adjusted to 1×10^3 cells per 100 μl. Aliquots of the cell suspension were incubated for 60 min at 37°C with dilutions of SP, ranging from 10^{-5} to 10^{-12} *M*, washed twice in medium and further diluted to give suspensions of 1, 5, 10, 20, 40, 80 and 120 cells per 10 μl.

An incubation chamber was constructed by coating the bottom of a 5-cm diameter round culture dish with a 2-mm layer of epoxy cement. Prior to setting, a 3-mm diameter well was formed in the center of the cement layer which acted as the cell culture chamber. The culture dish was placed into a Narishige micro-incubation chamber (Narishige, Greenvale, NY, USA) positioned on the stage of a Nikon inverted microscope. The micro-incubation chamber maintained the culture dish and its contents at 37°C in a 95% oxygen, 5% CO_2 atmosphere throughout the duration of the studies. The culture chamber was filled with 25 μl of mineral oil prior to seeding with 10 μl of one of the lymphocyte suspensions. These suspensions were placed under the oil using a Model M-6 micro-injection apparatus (Narishige).

2.4. Microdialysis and sample recovery

Microdialysis was accomplished using a basic CMA Microdialysis unit (CMA/Microdialysis, Acton, MA, USA) consisting of a Model CMA/102 microdialysis pump (equipped with a single syringe), a CMA/142 fraction collector, and a microdialysis probe with a 10 mm long, 0.5 mm OD PES membrane (CMA/20 microdialysis probe, 100 kDa cut-off). Positioning of the microdialysis probe was performed using a Model M-155 micro-manipulator (Narishige) which allowed accurate localization of the probe tip to either the surface of a single cell or the center of a multi-cell cluster. The cells were perfused with PBS at a flow-rate of 4 μl/min and the returned dialysis fluid was continuously collected. Each 5-min fraction was analyzed.

2.5. Fluorescent derivatization of samples

The protein content of each 20-μl fraction was measured by direct spectrophotometry at 260/280 nm using a Beckman DU5 spectrophotometer (Beckman, Palo Alto, CA, USA) equipped with an ultra-micro cuvet. Each sample was adjusted to 1 ng/ml by adding 0–1.3 μl of PBS prior to the addition of 10 ml of a 1 ng/ml solution of Cy5 (Research Organics, Cleveland, OH, USA) dissolved in 0.5 *M* carbonate buffer, pH 9.5. The mixture was placed into a small glass screw-capped container and mixed for 15 min at room temperature. Following this incubation, the solution was centrifuged at 10 000 *g* for 2 min and analyzed by ICE.

2.6. ICE analysis of microdialysis samples

Immunoaffinity electrophoresis [15] was performed on a modified ISCO 3850 capillary electrophoresis system (ISCO, Lincoln, NB, USA) by manually introducing approximately 50-nl samples into the capillary via the manual purge port and allowing the injected materials to remain in contact with the immobilized antibody coating of the capillary for 2 min. The capillary was then purged with two 100-μl washes of 100 m*M* phosphate buffer, pH 7.4, to remove all unbound materials (including unbound fluorochrome) prior to recovery and analysis of the bound analytes. The capillary was placed into buffer reservoirs containing 100 m*M* phosphate buffer, pH 1.5, and the electro-elution–separation performed at 100 μA constant current. Detection of the fluorochrome-labeled cytokines was achieved using a non-commercial LIF detector equipped with a 1-mW miniature diode laser module beam (650 nm, Edmund Scientific, Barrington, NJ, USA) focused at a 15° angle onto the capillary via a 50-mm focal length plano convex lens. The laser output was filtered through a 10-nm spatial filter and the emission collected and collimated using a parabolic reflector, A scatter mask was placed over the reflector which positioned the flow-cell of the capillary at the reflectors point of focus. The emission was

passed through a 671±10 nm interference filter prior to detection by a micro-CCD camera (Edmund Scientific) and the signals relayed to an image capture board in an IBM AT computer. Data was analyzed using ANELISA-R software (Man-Tech, Buffalo, NY, USA) and compared to standard curves constructed from known amounts of pure recombinant cytokines.

2.7. Chemiluminescence-enhanced immunoassay of microdialysis samples

The concentrations of the microdialysis recovered cytokines were measured by a modification of the technique previously described [8,17]. Briefly, the ICE-separated peaks were collected on a membrane fraction collector [17] and incubated overnight at 4°C with 200 μl of alkaline phosphatase-labeled anti-cytokine antibody (R&D Systems). The membrane was washed five times in 0.01 *M* phosphate buffered saline–0.01% Tween 20, pH 7.2, before adding 250-μl of a 25 m*M* solution of AMPPD chemiluminescent substrate (Tropix, Bedford, MA, USA). Following a 30-min incubation in the dark at room temperature, the chemiluminescent reaction product was read in a luminometer (Tropix) at 477 nm and analyzed in an identical manner as described for the ICE technique.

3. Results

Characterization of the ICE capillaries showed that approximately 85 ng of the FAb mixture was immobilized to the interior of the capillary. Saturation experiments using recombinant cytokines demonstrated that the immobilized FAb mixture was able to capture a maximum of 10.6 ng of IL-2, 12.9 ng of IL-4, 8.5 ng of IL-5, 10.3 ng of IL-10 and 12.7 ng of IL-12. Experiments using recombinant cytokines demonstrated that the different binding affinities of the five immobilized anti-cytokine FAb fragments produced a range in analyte retention which resulted in elution of the five cytokines in the following order: IL-2, IL-4, IL-10, IL-12 and IL-5 (Fig. 1). The ICE capillaries were stable for 6 weeks when kept at room temperature and could be used to perform approximately 200 analyses before appreciable deterioration in antibody activity was noted. Dilution studies demonstrated that the lower detection limits

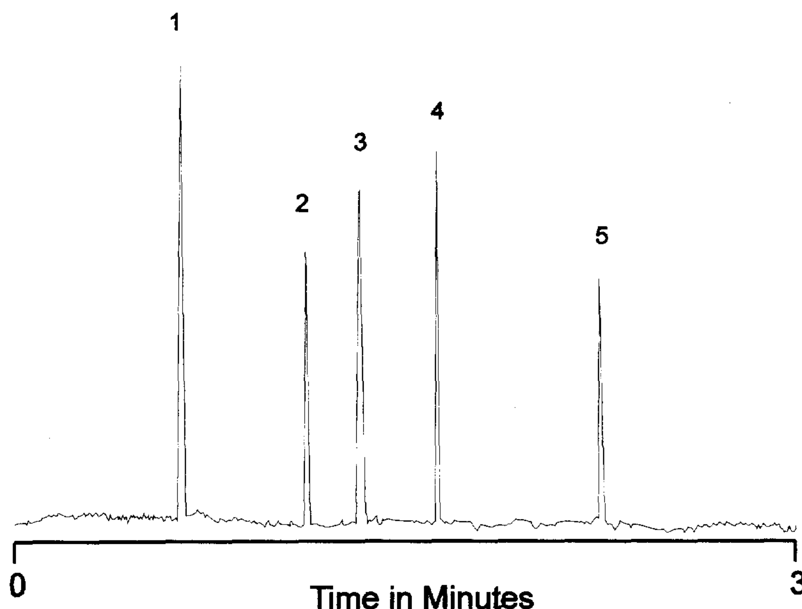

Fig. 1. Typical ICE electropherogram of recombinant cytokines recovered by microdialysis from cell-free micro-droplets. Peak 1=IL-2; peak 2=IL-4; peak 3=IL-10; peak 4=IL-12 and peak 5=IL-5.

of the ICE system were: 1.1 fg/μl for IL-2; 3.3 fg/μl for IL-4; 4.9 fg/μl for IL-5; 1.8 fg/μl for IL-10 and 2.7 fg/μl for IL-12.

The sensitivity of the ICE system was greatly enhanced by use of the red fluorochrome, Cy5, which is water-soluble and rapidly conjugates with free amino groups present in the target peptide. Additionally, this fluorochrome possesses a number of properties which allow detection of labeled molecules using relatively inexpensive diode laser excitation. The fluorochrome has an excitation maximum in the red portion of the spectrum at 652 nm. This closely matches the output wavelength of many commercially-available diode lasers thus allowing the fluorochrome to utilize approximately 85–90% of the available light energy. Additionally, the extremely high absorptivity of Cy5 (>200 000 M^{-1} cm^{-1}) allows the emission spectrum to be photometrically detected by a relatively simple CCD camera coupled with an image capture board.

The ability of microdialysis to recover secreted cytokines was examined by placing the dialysis probe into 25-μl droplets of medium in which known concentrations of recombinant cytokines had been dissolved. Microdialysis was performed for 60 min and samples collected continuously throughout the time period. The effect of flow-rate on the efficiency of the microdialysis probe to recover known amounts of recombinant cytokine was examined and the results are summarized in Table 1. When PBS alone was used as the dialysis fluid the maximum recovery was approximately 86% for all five cytokines but this situation improved drastically with the addition of 0.01% detergent. The greatest improvement was achieved with the addition of Brij 30 which increased the recovery to 95% for IL-4 and IL-5 and to 98% for the other three cytokines. Incubation of unstimulated T-lymphocytes in the presence of 0.01% detergent did not induce any detectable release of any of the five cytokines under investigation. The effects of these detergent additives on the efficiency of microdialysis recovery of IL-2 is summarized in Table 2.

Cell density greatly affected the ability of the microdialysis system to recover detectable concentrations of secreted cytokines. The system described in this study failed to detect cytokine release at the single cell level and required a minimum of 5–10 cells per culture in order to detect the presence of all five cytokines. This restriction was thought to be caused by the size of the dialysis probe rather than the efficiency of the system itself. Work is now in progress to redesign a microdialysis probe in order to study its ability to detect secreted materials from a single cell. Despite this restriction, the system could easily measure release of IL-2, IL-10 and IL-12 from cultures containing five lymphocytes. Recovery and detection of IL-4 and IL-5 required a minimum of 10 cells per culture, possibly due to the low concentrations of these cytokines produced by neuropeptide-stimulated lymphocytes. The effects of cell density on the recovery and measurement of the five cytokines under investigation are shown in Fig. 2.

The concentrations of SP required to induce a reproducible activation of T-lymphocytes was found

Table 1
Effect of dialysis flow-rate on 60 min recovery of recombinant cytokines from cell-free droplets

Flow-rate (μl/min)	Cytokine	Droplet concentration (fg/μl)	Recovery (fg/μl)
1	IL-2	20	13.4
	IL-4	20	8.6
	IL-5	20	10.3
	IL-10	20	11.5
	IL-12	20	10.9
2	IL-2	20	14.8
	IL-4	20	9.6
	IL-5	20	9.9
	IL-10	20	12.7
	IL-12	20	12.0
3	IL-2	20	15.6
	IL-4	20	15.2
	IL-5	20	13.8
	IL-10	20	15.8
	IL-12	20	14.7
4	IL-2	20	19.4
	IL-4	20	17.9
	IL-5	20	17.5
	IL-10	20	19.1
	IL-12	20	18.9
5	IL-2	20	16.3
	IL-4	20	16.1
	IL-5	20	15.2
	IL-10	20	16.5
	IL-12	20	13.9

Table 2
Effects of different detergent additives on the recovery of recombinant IL-2 from cell-free droplets

Additive	Amount of cytokine added (fg/μl)	n	Amount recovered (fg/μl)
No additive	10	5	8.6±1.6[a]
	20	5	17.2±3.3
0.01% Tween 20	10	5	8.9±2.0
	20	5	17.9±2.8
0.01% Nonidet	10	5	8.8±1.5
	20	5	18.0±2.4
0.01% Brij 30	10	5	9.7±0.5
	20	5	19.4±0.8

[a] Values expressed as mean±S.D.

to be 10^{-10} mol/ml (Table 3). Below this concentration the lymphocytes either failed to respond or they responded in an inconsistent manner. Higher concentrations of SP produced a variety of effects ranging from hyper-stimulation but no cytokine production to cell senescence and death.

ICE analysis of recovered micro-dialysis samples from SP-stimulated lymphocyte cultures produced the typical electropherograms shown in Fig. 3. In all three of the examples, the position of each peak was dependant upon (i) the binding affinity of the anti-cytokine antibody for its respective antigen, and (ii) on the electrophoretic mobility of the released molecule. The identity of each peak was verified by capturing it on a membrane fraction collector and characterizing the captured material by chemiluminescence-enhanced immunoassay. Comparison of the results obtained when cultures were analyzed by the microdialysis technique coupled to ICE detection and the chemiluminescence-enhanced immunoassay dem-

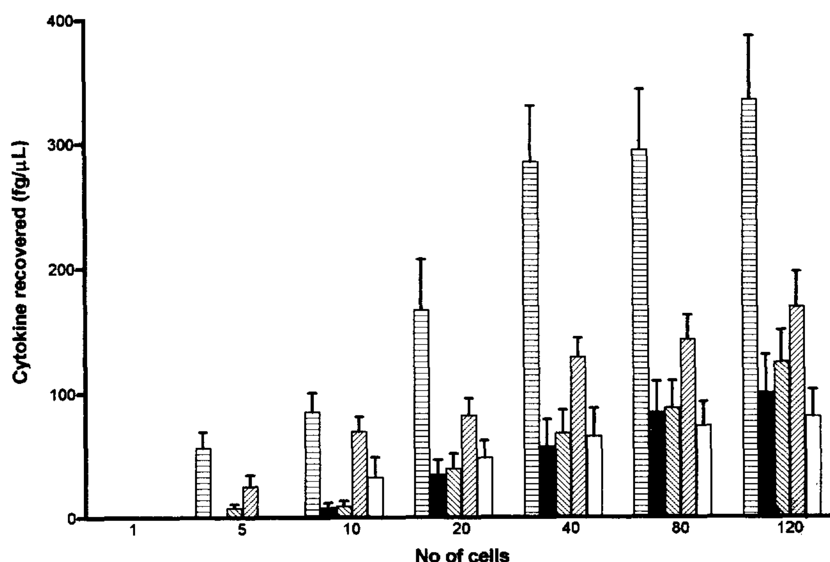

Fig. 2. Effects of cell culture density on the recovery and detection of different cytokines using microdialysis coupled to ICE detection. Horizontal striped bar=IL-2; solid bar=IL-4; right diagonally striped bar=IL-10; left diagonally striped bar=IL-12; solid bar=IL-5.

Table 3
The effects of different SP concentrations on cytokine secretion by 10-cell density cultures

SP concentration (mol/ml)	Cytokine detected	Maximum concentration detected (fg/μl)
10^{-6}	No cytokines detected	
10^{-8}	IL-2	38.6
	IL-10	24.9
	IL-12	16.4
10^{-10}	IL-2	89.8
	IL-4	25.2
	IL-5	29.6
	IL-10	73.4
	IL-12	61.3
10^{-12}	No cytokines detected	

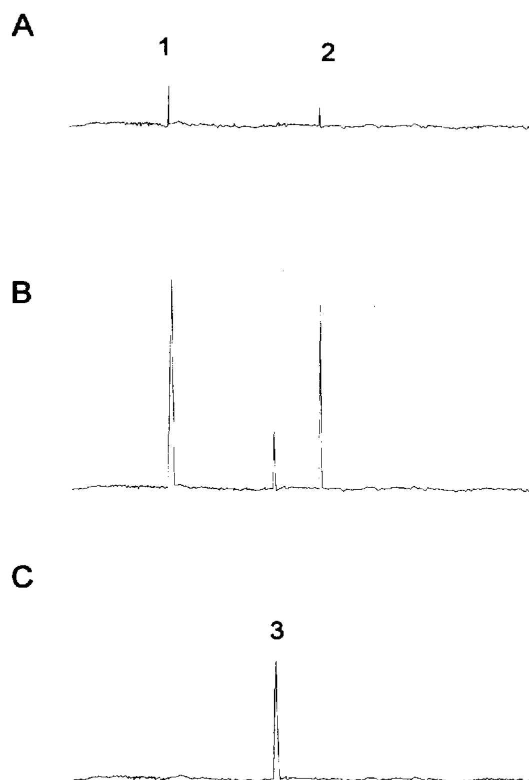

Fig. 3. ICE electropherograms of cytokine secretion by a 5-cell density culture following SP stimulation. (A) 1 min post-stimulation; (B) 10 min post-stimulation; (C) 20 min post-stimulation. Peak 1=IL-2; peak 2=IL-10; peak 3=IL-12.

onstrated a good correlation (r^2=0.999907; slope= 1.001356±0.001565 and y intercept=− 1.28674±0.148867) when the data were analyzed by linear regression using InStat (GraphPad Software, San Diego, CA, USA) statistical analysis program. The intra- and inter-assay coefficients of variation (C.V.) for the microdialysis–ICE technique were calculated based on measurement of five repetitive 10 fg/μl samples of each cytokine over five consecutive days. This study demonstrated intra-assay C.Vs of 2.69%, 3.81%, 3.66%, 2.93%, 2.75% for the recovery of IL-2, IL-4, IL-5, IL-10 and IL-12, respectively and inter-assay C.V.s of 3.95%, 4.16%, 4.44%, 3.72%, 4.11% were obtained for the same order of cytokines.

The time-course of SP stimulation of the target T-lymphocytes is given in Fig. 4. The data shown in this figure were taken from cultures with a cell density of 10 cells per culture as it was found that all five cytokines could be detected by the microdialysis–ICE system at this cell concentration. Within 1 min post-stimulation, low concentrations of IL-2 (16.4±5.5 fg/μl) and IL-12 (8.3±2.5 fg/μl) could be detected which rose to a maximum (116.8±35 fg/μl for IL-2 and 66.3±10 fg/μl for IL-12) at 10 min before they began to decline and became undetectable by 20 min. IL-10 became detectable (8.8±3 fg/μl) at 5 min and persisted throughout the duration of the study gradually becoming more concentrated as time passed (133.4±20.6 fg/μl at 60 min post-stimulation). In a

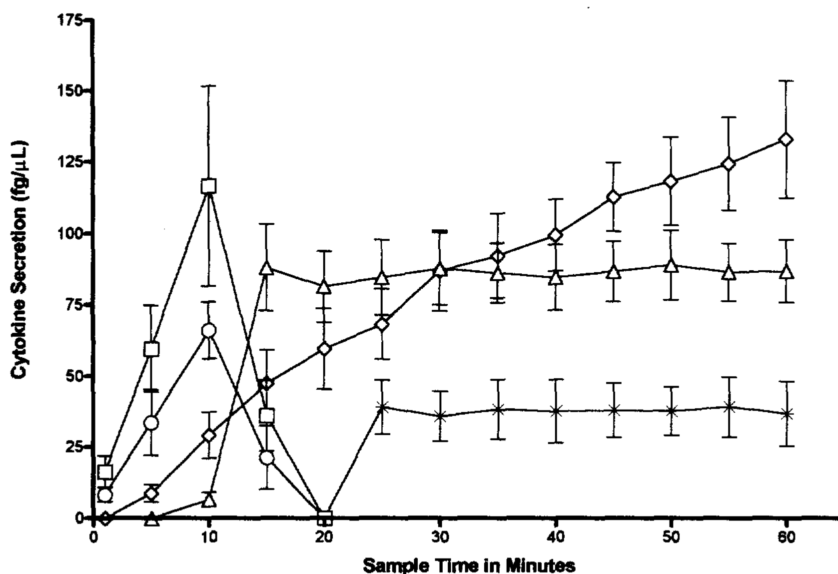

Fig. 4. Time-course of cytokine release by a 10-cell density culture following SP stimulation. (□) IL-2; (○) IL-12; (◇) IL-10; (△) IL-4; (*) IL-5.

similar manner, IL-4 was detected (6.6±2.5 fg/µl) at 10 min post-stimulation and also persisted throughout the study. However, this cytokine appeared to reach maximum release (88.3±15.2 fg/µl) at 15 min and remained at approximately that concentration throughout all subsequent sampling. IL-5 was not detected until 25 min post-stimulation (39.2±9.6 fg/µl) and was still present at the end of the study. There was no increase in production of this cytokine over the initial level detected in the following samples.

4. Discussion

Regulatory pathways controlling host defense mechanisms involve a number of accessory molecules, including cytokines [18], which are released following interaction with a variety of different stimuli. Interest has now centered on the role of neuronal intervention in immune reactivity, especially the role of neuropeptides in normal and pathological situations. The findings reported in this study agree with previous reports [4,19] that the neuropeptide, substance P, can specifically activate T-lymphocytes inducing the release of cytokines associated with specific immuno-regulatory pathways [18]. We have applied microdialysis to the recovery of secreted cytokines from low-density lymphocyte cultures in an attempt to study the effects of SP at the cellular level. The application of this procedure enabled us to continuously collect secreted materials from cultures with cell densities as low as 5–10 cells per culture over a reasonable period of time. Our findings suggest that although the initial responses of neuropeptide-activated lymphocytes indicated production of cytokines associated with the development of cell-mediated host responses (IL-2 and IL-12) [20], this response became attenuated within 20 min following stimulation. Continuous monitoring of the lymphocyte secretion products indicated a change in cytokine production associated with the development of an antibody-mediated response (IL-4 and IL-10). This latter response persisted throughout the remainder of the study indicating that SP activation of T-lymphocytes can produce profound regulatory effects [21] which affect host responses in the absence of specific antigen.

Detection and measurement of the recovered analytes was achieved by immunoaffinity CE coupled to LIF. This provided the degree of sensitivity required for analyzing molecules in extremely small

volume biological samples. CE is a suitable ultra-micro technique for monitoring biochemical events at the cellular and even sub-cellular level. Depending upon the method of detection, the procedure can efficiently separate 1–30 nl samples into multiple peaks and achieve detection levels in the attomole (10^{-18} *M*) range [22–24]. An example of the resolving power of CE was recently reported in which assessment of the concentration of carbonic anhydrase in a single red blood cell was made using mass spectrometry as the detection system [25]. CE can also be used as an analytical tool for measuring extra-cellular materials recovered by a variety of procedures including in-vivo microdialysis [10]. Additionally, it has been used in conjunction with a number of immunological techniques such as ultra-micro immunoassays and immunoaffinity separations of biological fluids [15].

Acknowledgments

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