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Discovery of endogenous catecholamines in lymphocytes and evidence for catecholamine regulation of lymphocyte function via an autocrine loop

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ABSTRACT **Evidence has been obtained that catecholamines and their metabolites are present in single lymphocytes and extracts of T- and B-cell clones by use of capillary electrophoresis with electrochemical detection. Pharmacological inhibition of tyrosine hydroxylase reduces observed catecholamine levels, suggesting catecholamine synthesis by lymphocytes. Intracellular dopamine levels are shown to be increased by extracellular dopamine, suggesting a cellular-uptake mechanism. Furthermore, incubation with either dopamine or L-dihydroxyphenylalanine, a precursor of dopamine, results in a dose-dependent inhibition of lymphocyte proliferation and differentiation. Together, these results suggest the presence of an autocrine loop whereby lymphocytes down-regulate their own activity.**

Lymphocytes crossing the blood–brain barrier appear to undergo a transformation that is involved in the experimental disease autoimmune encephalomyelitis, which has been used as a model for multiple sclerosis (1–3). The changes that occur to lymphocytes as they cross the blood–brain barrier are not at all clear. Various peptides have been implicated as potential modulators of lymphocyte function; however, interactions between lymphocytes found in the cerebrospinal fluid (CSF) and the nervous system might well involve classical neurotransmitters. Neurotransmitters have been shown to regulate the growth of nonneuronal cells by altering proliferation, differentiation, cell motility, and metamorphosis (4). This influence appears to occur via a receptor-mediated second-messenger system. CSF contains relatively low numbers of lymphocytes, and obtaining enough cells from human patients for analysis is difficult. Hence, it is useful to consider methods that can be used for qualitative and quantitative chemical analysis at the level of a single cell to study changes in lymphocytes crossing the blood–brain barrier.

Capillary electrophoresis is an emerging technique capable of rapidly determining multiple chemical species in picoliter and femtoliter biological samples, including single cells (5–10). This technique has been used successfully to develop quantitative analyses for chemicals in single whole nerve cells, including large invertebrate cells (8) and small mammalian erythrocytes (9). Capillary electrophoresis has been used to remove picoliter samples of cytoplasm from single large invertebrate neurons for separation and quantitative detection by capillary electrophoresis (10). Electrochemical detection is extremely sensitive with mass detection limits for capillary electrophoresis as low as 300 zmol (*vide infra*). In addition, capillary electrophoresis with electrochemical detection can routinely be used with capillaries as small as 2 μm i.d. (10, 11), which is important for analysis of volume-limited samples such as single cells.

In this paper, we describe the use of capillary electrophoresis with electrochemical detection in narrow-bore capillaries to examine the contents of single human lymphocytes and extracts of lymphocyte populations. In these experiments it has been discovered that CSF lymphocytes and cloned lymphocytes contain catecholamines, and catecholamines are shown to affect the proliferation and differentiation of lymphocyte populations in culture. CSF lymphocytes have been implicated in several diseases including autoimmune encephalomyelitis (1–3). In this scenario, a connection between glial cells and brain lymphocytes appears to be important in immunosurveillance of the central nervous system (12). In addition, a key immunological question after infection by human immunodeficiency virus type 1 is the mechanism by which human immunodeficiency virus type 1 decreases the population of $CD4^+$ T lymphocytes (13). Thus, information concerning the mechanisms by which lymphocytes regulate their populations could be extremely valuable to understanding the immune system and related diseases.

EXPERIMENTAL PROCEDURES

Preparation of CSF Lymphocytes and $CD4^+$ T- and B-Cell Extracts. CSF lymphocytes were prepared by centrifugation of 12 ml of cerebrospinal fluid at 100 × *g* for 20 min at 4°C. The upper 10 ml of CSF was drawn off and used for biochemical determinations; the lower 2 ml was used for cell counting and analysis by capillary electrophoresis.

Mononuclear cells were eluted from a prostate cancer tissue specimen after enzymatic digestion of the tissue as described (14, 15). T-cell clones were established by the limiting-dilution technique (16) in the presence of irradiated, autologous mononuclear feeder cells. Interleukin 2 (Amersham) and anti-CD3 antibodies (Ortho Diagnostics) were used to promote growth of the clones (17). Immunophenotyping was done by incubation with fluorescein isothiocyanate (FITC)-labeled anti-CD3 (Leu-4, Becton Dickinson) or with FITC-labeled anti-CD4 (Leu-3a) together with phycoerythrin-labeled anti-CD8 (Leu-2a). A FACScan flow cytometer (Becton Dickinson) was used for analysis. Cells were incubated for 1 hr with α-methyl-*p*-tyrosine (MeTyr) or dopamine at 37°C with 100% relative humidity and 7% CO_2 in air in a Forma Scientific incubator. B cells were grown autonomously. Extractions of cloned T- and B-lymphocyte populations were done by centrifugation, removal of supernatant, and then addition of 100 μl of 0.1 M perchloric acid/1 mM sodium EDTA/1 mM sodium sulfonate to the centrifugation pellet after washing twice with phosphate-buffered

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Abbreviations: CSF, cerebrospinal fluid; L-dopa, L-dihydroxyphenylalanine; DOPAC, dihydroxyphenylacetic acid; MeTyr, α-methyl-*p*-tyrosine; PBMC, peripheral blood mononuclear cells.

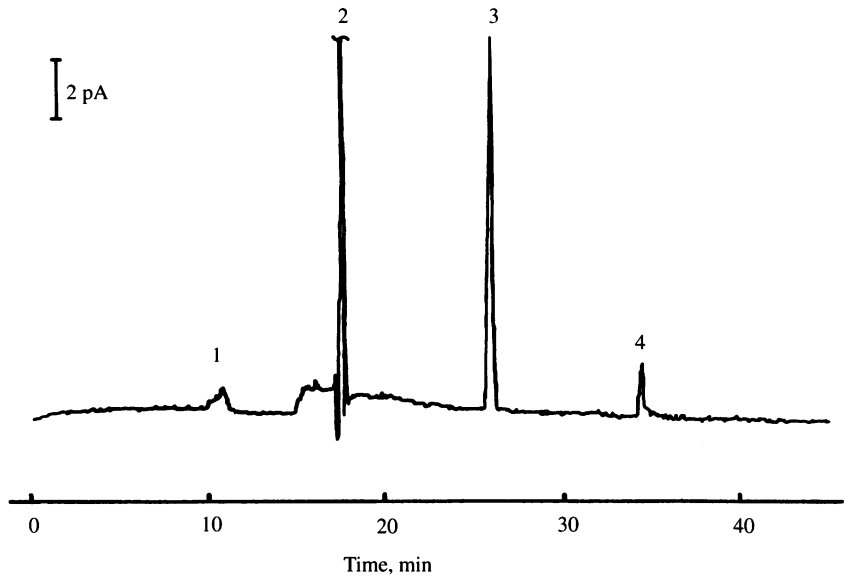

FIG. 1. Capillary electropherogram of a single human CSF lymphocyte. Conditions were as follows: separation capillary, 10-μm i.d., 80-cm length; buffer, 25 mM MES (pH 5.65); injection, 60 s at 1 kV to draw the lymphocyte into the capillary tip followed by a 15-s injection of digitonin to permeabilize the cell; separation potential, 25 kV; amperometric detection potential, 0.8 V vs. sodium-saturated calomel-reference electrode. Electrophoretic mobilities of the major peaks correspond to the calculated electrophoretic mobilities of dopamine (peak 1), a neutral species (peak 2), uric acid (peak 3), and DOPAC (peak 4).

saline. This mixture was ultrasonicated for 1 min on ice, and the extract was centrifuged for 30 min (35,000 × *g*) at 4°C.

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Ficoll/Hypaque (Sigma) density gradient and resuspended in Iscove's medium (GIBCO)/5% fetal calf serum (GIBCO).

Capillary Electrophoresis Apparatus. The system used for coupling electrochemical detection to capillary electrophoresis in small-bore capillaries was similar to that described in ref. 5. Briefly, the apparatus consisted of a segmented capillary joined by a porous glass joint. This segmented capillary was placed between two buffer reservoirs with high voltage applied at the end containing the microinjector, and the reservoir containing the porous joint was held at ground potential. Fused silica capillaries with 10-μm i.d. were obtained from Polymicro Technologies (Phoenix). Easily oxidized analytes were detected in the amperometric mode with a two-electrode configuration. The carbon-fiber microelectrode was inserted into the end of the electrophoresis capillary and held at 0.8 V vs. a sodium-saturated calomel electrode. A capillary with an etched tip (HF) as described (8, 10) was used to pull a single cell into the capillary by electroosmotic flow. All errors are reported as SEMs.

Reagents. 2-(*N*-Morpholino)ethanesulfonic acid (MES), dopamine, L-dihydroxyphenylalanine (L-dopa), and dihydroxyphenylacetic acid (DOPAC) were obtained from Sigma and used in the form received. The electrophoresis buffer was 25 mM MES adjusted to pH 5.65 with NaOH. Calibration standards were prepared as 10 mM stock solution in perchloric acid and diluted to the desired concentration in electrophoresis buffer. HF was obtained as a 40% aqueous solution from Aldrich.

RESULTS AND DISCUSSION

Single-Cell Analysis. An electropherogram for a single lymphocyte, in this case removed from the CSF of a human patient, is shown in Fig. 1. This separation contains a peak (peak 1) at a migration time of 10.75 min that has tentatively been identified as dopamine; this identification is based on electrophoretic mobility, as described (8, 10). However, this peak is broader than normal for dopamine, which might indicate the presence of other amines with similar electrophoretic mobilities, including norepinephrine, epinephrine, or serotonin. Peak 2 at 17.62 min corresponds to the rate of bulk flow through the capillary and represents the elution of

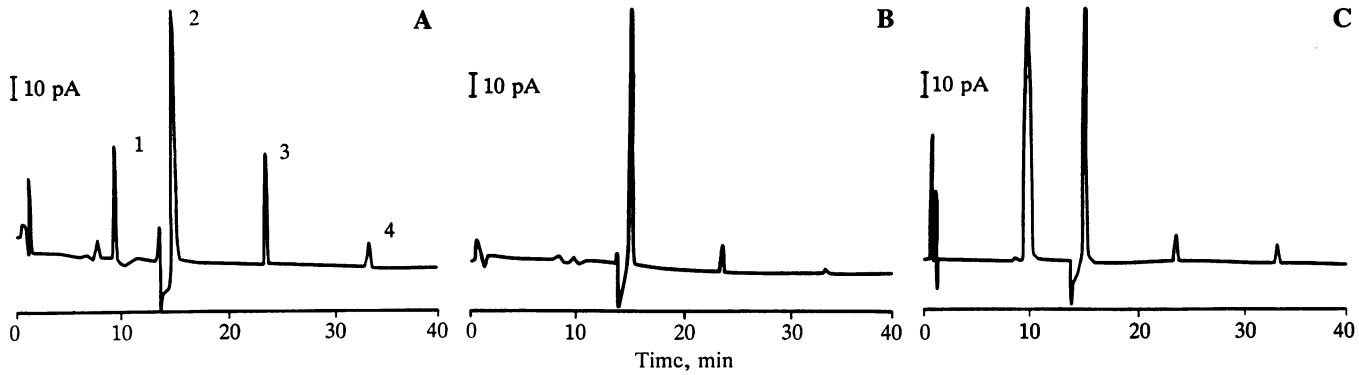

FIG. 2. Separation and detection of easily oxidized species in lymphocyte extracts by capillary electrophoresis. The capillary electrophoresis system was the same as in Fig. 1. Injection was 5 s at 25 kV. (*A*) Electropherogram of extract of CD4+ T-lymphocyte clones. (*B*) Electropherogram of extract of CD4+ T-lymphocyte clones after incubation in 10 μM MeTyr for 1 hr. (*C*) Electropherogram of extract of CD4+ T-lymphocyte clones after incubation in 10 μM dopamine for 1 hr. Peak identities are the same as for Fig. 1.

nonionic species. The peak at 25.90 min (peak 3) corresponds to uric acid. Finally, the peak at 34.50 min (peak 4) corresponds to DOPAC. As a control, an electropherogram of the cell medium has been injected with an equivalent injection time, and this electropherogram shows only a small peak at 17.6 min (data not shown) corresponding to a neutral easily oxidized species.

To our knowledge catecholamines have not before been reported to be present in lymphocytes. It is difficult to positively identify which catecholamine is present in these cells by capillary electrophoresis, as dopamine, norepinephrine, and epinephrine all have similar, although not identical, electrophoretic mobilities (10). However, the presence of a peak with a mobility similar to DOPAC, a primary metabolite of dopamine, strongly suggests that these cells, indeed, contain dopamine and do not contain norepinephrine or epinephrine.

Because lymphocytes obtained directly from the human body may have accumulated dopamine by active transport rather than by *in vivo* synthesis, we next examined long-term cloned $CD4^+$ T cells and B cells for the presence of catecholamines. Electropherograms containing the catecholamine and DOPAC peaks are obtained for the single-cell analysis of these cloned cells (data not shown), indicating that catecholamines are, indeed, produced and stored by lymphocytes. This result is discussed in the next section, where experiments concerning the effects of a synthesis inhibitor on catecholamine levels in extracts of cloned lymphocytes are described.

Catecholamine levels in single lymphocytes have been quantified by direct comparison with standard electropherograms run before and after the cell experiments. The peak corresponding to catecholamine has been averaged for experiments on CSF lymphocytes and monoclonal $CD4^+$ T cells. CSF lymphocytes are seen to contain 2.3 ± 1.7 amol ($n = 3$) of catecholamine, and cloned $CD4^+$ T cells are seen to contain 31 ± 29 amol ($n = 3$) of catecholamine. In addition, a single cloned B cell has been examined by capillary electrophoresis. For this one cloned B cell, we observed 310 zmol (3.1×10^{-19} mol) of catecholamine.

Evaluation of the concentration of catecholamine in lymphocytes is important to understanding its functional role in this system. Capillary electrophoresis with electrochemical detection provides an extremely sensitive method for single-cell analysis of easily oxidized substances. Femtomole levels of catecholamines have been quantitated in single adrenal cells by use of microbore liquid chromatography (18). In addition, attomole levels of proteins and inorganic ions have been determined in single human erythrocytes by capillary electrophoresis with laser-induced fluorescence detection (9, 19). The methodology described in this paper allows zeptomole levels of catecholamines to be determined and has allowed the discovery of catecholamines in lymphocytes. An estimate of the cellular concentration of the catecholamines in these cells could be useful. If the volume of a single lymphocyte is approximated at 180 fl and the catecholamines present are assumed to be evenly distributed in an individual lymphocyte, the catecholamine concentrations calculated from the average total moles observed for $CD4^+$ T cells (31 amol), CSF lymphocytes (2.3 amol), and the one B cell (310 zmol) correspond to 1.7×10^{-4} M, 1.3×10^{-5} M, and 1.7×10^{-6} M, respectively.

Analysis of Extracts of Lymphocyte Clonal Lines. Catecholamine levels have also been quantitated in extracts of $CD4^+$ T-cell populations by capillary electrophoresis. The average catecholamine level in the extracts (1.8 ± 1.0 amol per cell; $n = 13$; SEM) is within the range of catecholamine levels found in single $CD4^+$ T cells. Owing to apparent biological variance, catecholamine levels vary from a low of 110 zmol to a high of 20 amol per cell for the 13 extracts examined, each containing between 1 and 12 million cells obtained from eight different T-cell clones. The precision is better when extracts are examined for different plates of the same clone (one clone provided levels at 880 ± 430 zmol per cell; $n = 3$). In addition,

Table 1. Catechol levels in extracts of cloned lymphocytes

	Catecholamine peak, amol	DOPAC, amol	*n*
Control	1.8 ± 1.0	2.2 ± 1.3	13
MeTyr incubation	0.19 ± 0.11	0.57*	3
Dopamine incubation	89.8	236	1

Cells were incubated in either 10 μM MeTyr or 10 μM dopamine for 1 hr. Extraction volumes were 100 μl, and an average of 340 pl of each extract was injected onto the electrophoresis capillary. The fraction of extract volume injected was used with the total cell count to calculate the level of each substance per cell for each incubation. Errors are given as SEM. The catecholamine is assumed to be dopamine.

*DOPAC was not seen in the electropherograms for two of three extracts incubated with MeTyr.

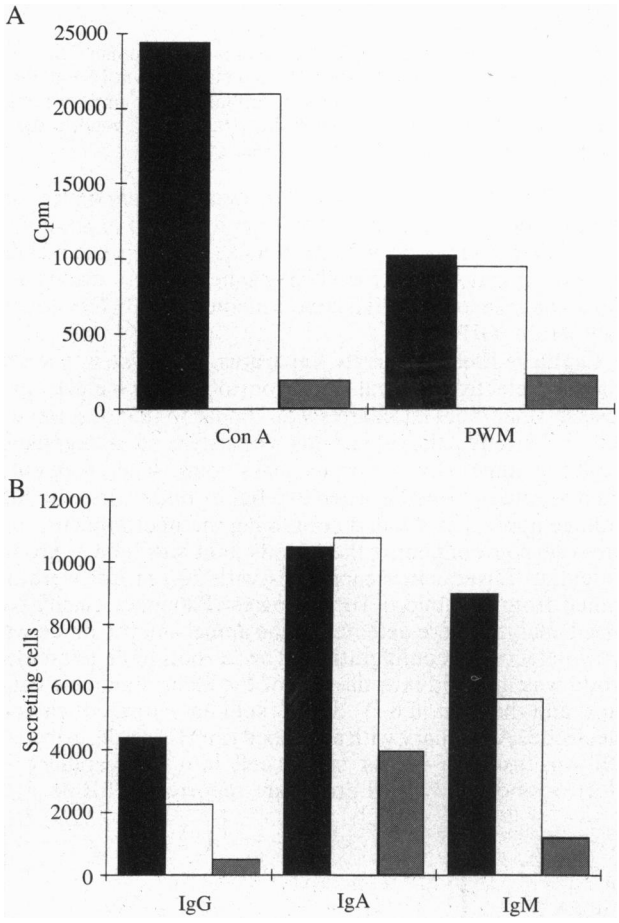

FIG. 3. Effect of L-dopa on proliferation and differentiation of human lymphocytes. (*A*) To evaluate the effect of L-dopa on proliferative responses, PBMC were incubated for 72 hr with T-cell mitogen Con A at 20 μg/ml or the B-cell mitogen pokeweek mitogen (PWM) at 10 μg/ml. Proliferative responses were measured by incorporation of [^{3}H]thymidine and expressed as cpm. ■, Control; □, 10 μM L-dopa; ▨, 100 μM L-dopa. (*B*) To assess the effect of L-dopa on differentiation of B lymphocytes, PBMC were incubated for 6 days with PWM at 10 μg/ml and varied concentrations of L-dopa. The cells were subsequently washed, recounted, and analyzed by the ELISPOT assay (17) with respect to frequencies of immunoglobulin-producing cells of IgG, IgA, and IgM isotypes. Results are expressed as numbers of immunoglobulin-secreting cells per 10^6 PBMC. ■, Control; □, 10 μM L-dopa; ▨, 100 μM L-dopa. All experiments were done in triplicate, and in all cases the relative SD was <10%.

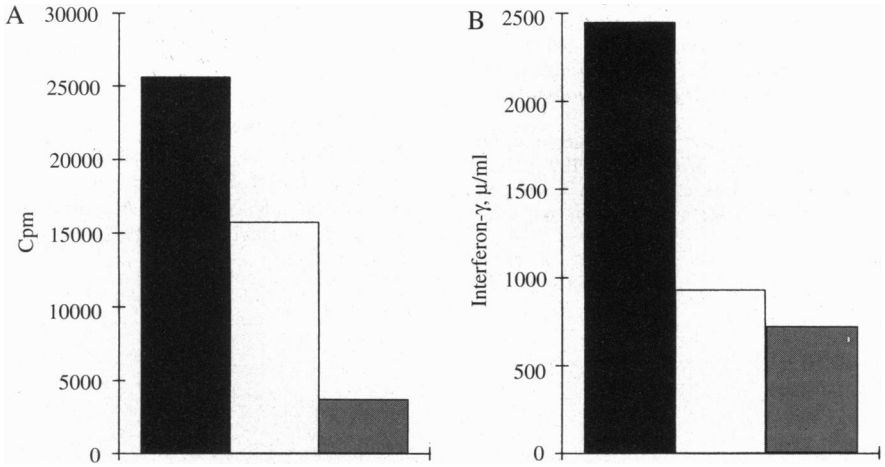

FIG. 4. Effect of dopamine on proliferation and differentiation of human lymphocytes. (*A*) Proliferative responses for cells incubated with Con A. Conditions were the same as for Fig. 3, except that cells were incubated with dopamine instead of L-dopa. (*B*) Effect of dopamine on differentiation of T lymphocytes as measured by synthesis of interferon-γ in Con A-stimulated cell cultures (72 hr). Incubation with 500 μM dopamine eliminated interferon-γ synthesis completely (data not shown). ■, Control; □, 10 μM dopamine; ▨, 100 μM dopamine. All experiments were done in triplicate, and in all cases the relative SD was <10%.

extracts of three different B-cell clones have been examined. The average catecholamine level in extracts of B cells is 2.0 ± 1.5 amol per cell ($n = 3$). Variation among single lymphocytes and different clones is expected, and the methods presented provide an excellent way to examine these differences.

To study the uptake and biosynthesis of catecholamines in $CD4^+$ T lymphocytes, we measured the effects of incubating T-cell clones with either dopamine or the dopamine synthesis inhibitor MeTyr. After incubation, the cultured cells were extracted, and a sample was examined by capillary electrophoresis (Fig. 2). A quantitative summary of catecholamine and DOPAC levels in lymphocyte extracts is provided in Table 1. After incubation with dopamine, a large increase in the level of catecholamine per cell is observed. Conversely, incubation with MeTyr decreases the level of catecholamine in cloned $CD4^+$ T lymphocytes. These data strongly suggest that the catecholamine peak is, indeed, dopamine and that this catecholamine is both synthesized and accumulated by lymphocytes. Experiments demonstrating an effective displacement of spiperone with the highly selective uptake inhibitor GBR 12909 have been used to suggest the presence of a dopamine transporter in peripheral lymphocytes (20) and are completely consistent with our data.

Effect of Catecholamine Levels on Lymphocyte Function. To examine the functional role for catecholamines in lymphocytes, PBMC were incubated *in vitro* with L-dopa and dopamine. L-Dopa clearly exerts a dose-dependent inhibition of both lymphocyte proliferation and differentiation (Fig. 3). However, an even larger dose-dependent inhibition of lymphocyte proliferation and differentiation is observed when cells are incubated with dopamine (Fig. 4). In fact, incubation with dopamine at concentrations from 10 μM to 500 μM completely abolishes the production of antibodies by B cells (data not shown). A dose-dependent effect is also observed for interferon-γ synthesis by T cells when incubated in dopamine (Fig. 4*B*). At the higher concentrations used, some effect from nonspecific reduction–oxidation reactions might be occurring; however, the lower end of these concentrations is consistent with the dopamine levels found in the extracellular fluid after neuronal stimulation (21). The combined data, showing the presence of catecholamines in lymphocytes and the effect of L-dopa and dopamine on lymphocyte proliferation and differentiation, indicate that catecholamines produced by lymphocytes act in an autocrine or paracrine way and are important regulatory molecules and, thus, are potentially important during an ongoing immune response. This hypothesis is consistent with findings that immune system cells carry β-adrenergic (22) and dopaminergic (23) receptors, which are a prerequisite for subsequent interaction with catecholamines.

These results emphasize the discovery of catecholamines in single CSF lymphocytes and a functional role for catecholamines involving the control of T and B cells. Regulation of lymphocyte function by catecholamines could prove to be an important part of immune activation in the nervous system. Much work is required to further examine this aspect of lymphocyte regulation.

Epstein–Barr virus-transformed human B-cell clones Raji, Namalva, and DG 75 were obtained from Dr. Lars Rymo, Department of Medical Chemistry, The Faculty of Medicine, Göteborg University. We gratefully acknowledge discussions and comments from S. Douglass Gilman. This work was supported, in part, by the Swedish Medical Research Council, the Faculty of Medicine, Göteborg University, Sweden, the U.S. National Institutes of Health and the U.S. National Science Foundation. A.E. is a Camille and Henry Dreyfus Teacher-Scholar.

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