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REGULAR ARTICLE

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Ultrastructural localization of tyrosine hydroxylase in human peripheral blood mononuclear cells: effect of stimulation with phytohaemagglutinin

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Abstract Using immunocytochemistry coupled to fluorescence and electron microscopy, we investigated the expression and ultrastructural localization of tyrosine hydroxylase (TH, EC 1.14.16.2), the rate-limiting enzyme in the biosynthesis of catecholamines, in human peripheral blood mononuclear cells (PBMCs), with PC12 cells as positive controls. In unstimulated PBMCs, TH-specific immunoreactivity was localized to the plasma membrane. However, after stimulation with the polyclonal mitogen phytohaemagglutinin (PHA), TH immunoreactivity was almost completely localized to electron-dense cytoplasmic granules, which resembled those found in PC12. TH-positive granules, however, were larger (300–500 nm) than in PC12 cells (100–200 nm). Flow cytometry analysis of TH expression showed about 46–50% positive cells in unstimulated PBMCs and in PHA-stimulated PBMCs in the G0/G1 phase of the cell cycle, but more than 80% positive cells in PHA-stimulated PBMCs in the S+G2/M phase. In agreement with previous observations, PHA stimulation also induced de novo expression of TH mRNA as well as increased intracellular catecholamine content, suggesting the occurrence of TH upregulation at the level of both gene expression and enzyme activity. The ultrastructural localization of TH in human PBMCs seems therefore regulated by cell stimulation and related to the functional activity of the enzyme.

Keywords Peripheral blood mononuclear cells · Tyrosine hydroxylase · Catecholamines · Ultrastructure · Human

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Introduction

Although it is well established that sympathoadrenergic mechanisms play a major role in the cross-talk between the nervous system and the immune system (Friedman and Irwin 1997; Elenkov et al. 2000), compelling evidence that immune system cells themselves produce catecholamines was given only a few years ago (Bergquist et al. 1994; Musso et al. 1996; Marino et al. 1999). Immune-cell-derived catecholamines may be relevant not only for the neuroimmune network (Friedman and Irwin 1997; Elenkov et al. 2000), but also as autocrine/paracrine factors, as suggested by the ability of adrenergic antagonists to regulate macrophage production of tumor necrosis factor-α (Spengler et al. 1994).

Pharmacological evidence suggests that catecholamine synthesis in immune cells is likely to occur through the classical sympathoadrenergic pathways. In the adrenal medulla and in catecholaminergic neurons tyrosine hydroxylase (TH, EC 1.14.16.2) is the first and rate-limiting enzyme in catecholamine synthesis (see, e.g. Masserano et al. 1989). Inhibition of TH reduces intracellular catecholamine levels in mouse lymphocytes (Joseffson et al. 1996), macrophages (Spengler et al. 1994), and mast cells (Freeman et al. 2001), in human

lymphocytes (Bergquist et al. 1994), in peripheral blood mononuclear cells (PBMCs; Musso et al. 1996; Marino et al. 1999; Cosentino et al. 2000), and even in hematopoietic cell lines (Cosentino et al. 2000). Since TH controls the rate of catecholamine synthesis also in immune cells, studying the regulatory mechanisms of TH in these cells should contribute to a better understanding of the role(s) of immune-cell-derived catecholamines.

Evidence exists that in adrenal medulla, sympathetic nerves and brain (notably in the striatum), TH may exist in a soluble, less active form and in a membrane-bound, more active form. The ultrastructural localization of TH has been suggested to play a role in the regulation of catecholamine synthesis. In rat adrenal medulla, the stress-induced activation of TH was associated with a shift in the subcellular distribution of the enzyme from the cytosol to membranous structures. In agreement with these results, a marked increase in the enzyme activity was produced by incubation of TH with isolated chromaffin granule membranes or with the cytoskeletal component G-actin. This issue has been extensively reviewed by Masserano and coworkers (1989).

Few data exist at present concerning the ultrastructural localization of TH in immune cells. The expression of TH was shown by flow cytometry in various immune cell lines (Tsao et al. 1998) and recently by confocal laser microscopy TH immunoreactivity was found to be diffused to cytoplasm and plasma membrane of human lymphocytes (Amenta et al. 2001). Human PBMCs, however, were previously reported to express no detectable levels of TH mRNA (Miyajima et al. 1995), a finding which indirectly suggests that in these cells the enzyme may be highly stable. Indeed, increasing the intrinsic activity of TH has been shown to decrease the half-life of the enzyme (reviewed by Kumer and Vrana 1996). In this regard, some of us have recently shown that stimulation of human PBMCs with the polyclonal mitogen phytohaemagglutinin (PHA) induces a dramatic increase in the production of catecholamines, which is preceded by the expression of TH mRNA (Cosentino et al. 2002). Based on these findings, it is conceivable that stimulation of PBMCs leads to increased turnover of TH as a result of increased activity of the enzyme.

The present study was therefore undertaken to examine the ultrastructural localization of TH in human PBMCs and to test the possibility that in PBMCs TH activation may correspond to a subcellular redistribution of the enzyme.

Materials and methods

Cells

Human PBMCs were isolated from venous blood obtained from healthy volunteers using heparinized tubes. Whole blood was allowed to sediment on dextran at 37°C for 30 min. Supernatant was recovered and PBMCs were separated by density-gradient centrifugation using Ficoll-Paque Plus. Cells were then washed 2 times in NaCl 0.15 M and resuspended at a final concentration of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine and 100 U/ml penicillin-streptomycin, at 37°C, in a moist atmosphere of 5% CO_2. Typical PBMC preparations contained about 80% lymphocytes and 16% monocytes. Cell viability, assessed by the trypan blue exclusion test, was always >99%. PBMCs were cultured for 48 h in the presence of PHA at a concentration of 10 μg/ml, a treatment which in previous experiments was shown to induce a maximal increase in intracellular catecholamines (Cosentino et al. 2002). Rat pheochromocytoma cells (PC12) used as positive controls were obtained from the Interlab Cell Line Collection of the National Institute for Cancer Research/Advanced Biotechnology Center of Genoa, Italy. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal horse serum, 5% heat-inactivated fetal calf serum, 2 mM glutamine, and 100 U/ml penicillin-streptomycin at 37°C in a moist atmosphere of 5% CO_2.

Light-microscopic immunocytochemistry

Cells were smeared, air dried and fixed in 70% ethanol solution. After 1 h permanence in phosphate-buffered saline (PBS) 0.1 M containing 1% bovine serum albumin and 0.03% saponin, the smears were incubated with mouse anti-TH monoclonal antibody (Chemicon, Temecula, CA) 1:300 overnight at 4°C in a dark humid chamber. After three washes in PBS the slides were incubated with goat anti-mouse IgG fluorescein isothiocyanate (FITC)-labelled antibody (Molecular Probes Inc., Eugene, OR) 1:200 for 1 h at room temperature. Finally, the cells were washed and treated with streptavidin FITC-conjugated (Caltag Laboratories) 1 μg/10^6 cells for 30 min at 4°C in the dark. The slides were then washed 3 times in PBS and mounted in glycerol. All observations were performed with a Nikon Eclipse E600 microscope at 450–490 nM wavelength.

Electron-microscopic immunocytochemistry

After centrifugation at 1,000 *g* for 5 min, cells were fixed in 0.5% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M PBS (pH 7.6) for 1 h at room temperature. Cells were then fixed in 2.5% buffered glutaraldehyde for 1 h, postfixed in PBS with added OsO_4 1% and stained with lead citrate and uranyl acetate according to Reynolds (1963) to enhance image definition. Lead/uranyl staining, however, was omitted in preparations subsequently labelled for TH, to avoid false positivity. Before labelling for TH, cells were washed 3 times in PBS treated with H_2O_2 3% in PBS for 30 min to inhibit endogenous peroxidases. Cells were then incubated with mouse anti-TH monoclonal antibody (Chemicon, Temecula, CA) 1:200 overnight at 4°C in PBS containing saponin 0.03%. Normal goat serum 1% was always added to prevent non-specific binding of the antibody. The anti-TH antibody was demonstrated by the avidin-biotin-peroxidase method (ABC kit, Vector Laboratories, Burlingame, CA) in TRIS buffer 0.05 M (pH 7.6) with added H_2O_2 0.3% for 10 min at room temperature, using diaminobenzidine as a chromogen. Both TH-labelled and unlabelled cells were further fixed in glutaraldehyde 2.5% for 1 h, postfixed in OsO_4 1%, briefly dehydrated in graded ethanol and propylene oxide and embedded in Epon 812. Ultrathin sections were cut, fixed in 2.5% buffered glutaraldehyde for 1 h, postfixed in OsO_4 1% and observed without overstaining under an electron microscope (Jem-1010, JEOL, Akishima, Japan). Specificity of labelling was assessed by either omitting the incubation step with the anti-TH antibody (negative control) or substituting the anti-TH antibody with a mouse monoclonal IgG2a with irrelevant specificity (Immunotech, Marseille, France; isotypic control). In neither case could significant diaminobenzidine deposition be observed.

Flow cytometry analysis of TH expression

TH in human PBMCs was labelled by using an indirect immunofluorescence method. Briefly, cells were centrifuged, resuspended

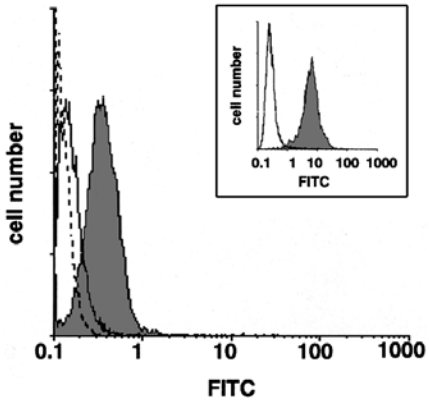

Fig. 1 Representative flow cytometric analysis of TH expression in human PBMCs. TH was labelled by using an indirect immunofluorescence method with the biotin-streptavidin system for the amplification of the signal. Data display TH expression as fluorescence intensity of FITC in arbitrary units. *Filled histogram* TH-labelled cells (i.e. cells labelled with mouse anti-TH mAb followed by goat anti-mouse IgG-biotin and streptavidin-FITC); *open histograms with solid line* negative control (i.e. cells labelled with goat anti-mouse IgG-biotin and streptavidin-FITC only); *open histogram with dotted line* isotypic control (i.e. cells labelled with a mouse monoclonal IgG2a with irrelevant specificity followed by goat anti-mouse IgG-biotin and streptavidin-FITC). *Inset:* Flow cytometric analysis of TH expression in PC12 cells (*filled histogram* TH-labelled cells; *open histogram* negative control). For further details, see "Materials and methods"

in phosphate-buffered saline, fixed and permeabilized with ethanol 70% (4°C, 40 min). Cells were then rinsed and incubated with mouse anti-TH monoclonal antibody (0.8 µg/10^6 cells; Chemicon, Temecula, CA) for 30 min at 4°C. Subsequently, the cells were washed and treated with biotin-labelled goat anti-mouse antibody (2 µg/10^6 cells; Caltag Laboratories, Burlingame, CA) for 30 min at 4°C. Finally, the cells were washed and treated with streptavidin FITC conjugated (1 µg/10^6 cells, Caltag Laboratories, Burlingame, CA) for 30 min at 4°C in the dark. Since immune cells may express receptors for the Fc of antibodies, preliminary experiments with an isotypic control (mouse monoclonal IgG2a with irrelevant specificity, Immunotech, Marseille, France) were performed on human PBMCs to exclude non-specific binding by the anti-TH antibody. In each analysis, samples of the same specimens were processed omitting the incubation step with the anti-TH antibody and used as a negative control, and 10,000 cells/sample were analysed. A typical flow cytometric analysis of TH expression in unstimulated PBMCs (and, for comparison, in PC12 cells) is shown in Fig. 1. In some experiments the expression of TH was analysed together with cell cycle. To this end, after TH labelling cells were washed and incubated with 1 ml PBS containing propidium iodide (PI, 50 µg/ml; Sigma-Aldrich Inc., St. Louis, MI) and RNAse (0.2 mg/ml; Sigma-Aldrich Inc., St. Louis, MI) for 1 h at 4°C in the dark, and 25,000 cells/sample were analysed. The cell-associated fluorescence analysis was performed by using a Coulter Epics Elite ESP flow cytometer (Beckman-Coulter, Miami, FL). Cell fluorescence was quantified by using the mean channel fluorescence intensity (MFI), i.e. the median value of fluorescence intensity distribution. Results were then expressed both as MFI and as MFI ratio (i.e. MFI of anti-TH antibody-treated cells/MFI of negative controls).

RNA isolation and RT-PCR analysis of tyrosine hydroxylase mRNA

Total RNA was extracted from 1×10^6 PBMCs using the Perfect RNA Eukaryotic Mini kit (Eppendorf, Hamburg, Germany). The kit uses a chaotropic guanidinium isothiocyanate solution for cell lysis and rapid inactivation of cellular RNAses. RNA is subsequently bound to the matrix of the column, washed to remove contaminants and then eluted with molecular biology grade water. The amount of extracted RNA was estimated by spectrophotometry at 260 nm. Total RNA was reverse transcribed and cDNA was amplified using a one-step reverse transcriptase polymerase chain reaction (RT-PCR) reaction kit (Finnzymes, Espoo, Finland). Twenty-five cycles of PCR were then performed according to the following steps: 48°C, 30 min (once); 94°C, 1 min; 55°C, 1 min; and 72°C, 2 min. At the end, the reaction mixture was kept for 5 min at 72°C and finally chilled at –4°C until analysis, which was performed on a 10-µl aliquot of the PCR product by electrophoretical separation on a 2% agarose gel and subsequent visualization by ethidium bromide staining (Biorad, Hercules, CA). For selection of the primers, we referred to the National Center for Biotechnology Information database. The primers' sequences were as follows: 5′-primer, sense (exon 5) 5′-TGTCAGAGCTGGACAAGTGT-3′, and 3′-primer, antisense (exon 8) 5′-GATATTGTCTTCCCGGTAGC-3′. In the presence of TH mRNA, a 299-basepair cDNA fragment was expected to be amplified.

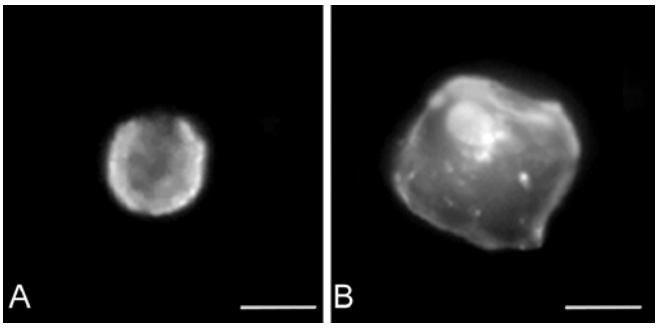

Fig. 2A, B Light micrographs of human PBMCs labelled for TH. In the absence of stimulation **(A)**, about 50% of the cells showed specific immunofluorescence for TH in the outer cytoplasm near the cell membrane. However, after stimulation with PHA **(B)**, membrane-associated fluorescence disappeared and a similar percentage of cells exhibited diffused TH-specific immunofluorescence to the cytoplasm. *Scale bars* 10 µm

HPLC-ED analysis of intracellular catecholamines

Intracellular catecholamines were assayed by high-performance liquid chromatography (HPLC) with electrochemical detection (ED) according to a previously described method (Marino et al. 1997).

Results

Light-microscopic immunocytochemistry

In both unstimulated and PHA-stimulated PBMCs, specific immunofluorescence for TH was evident in about 50% of the cells. However, in unstimulated cells fluorescence was confined to the cell membrane while in PHA-stimulated cells it was also present in the cytoplasm (Fig. 2).

Electron-microscopic immunocytochemistry

In agreement with previous classical observations (Bessis 1972), unstimulated PMBCs usually showed a large,

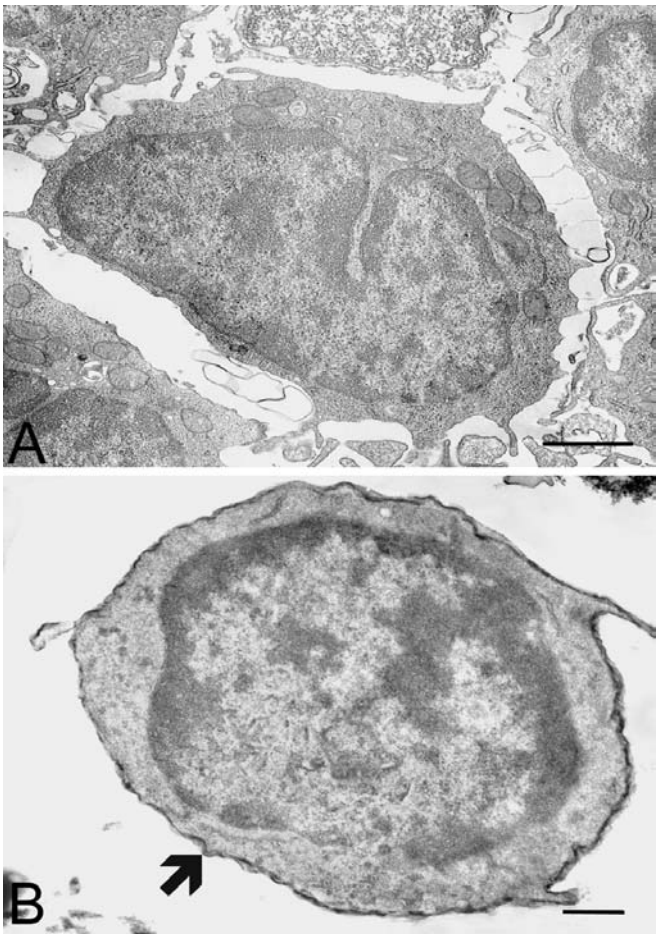

Fig. 3A, B Electron micrographs of human PBMCs. **A** Cell stained with lead citrate and uranyl acetate to enhance image definition; **B** unstained cell labelled for TH. In TH-positive cells, marked electron-dense positivity was visible near the plasma membrane and in the underlying thin cisternae pertaining to the smooth reticulum (*arrow*). Lead/uranyl staining was omitted to avoid false positivity. *Scale bars* 1 μm

roundish nucleus surrounded by scarce cytoplasm, with various mitochondria and free ribosomes. In some sections, rough reticulum and Golgi apparatus could be observed (Fig. 3A). In these cells, specific immunoreactivity for TH was evident as an electron-dense positivity just under the plasma membrane and in very thin cisternae pertaining to the smooth reticulum underlying the plasma membrane (Fig. 3B).

After PHA stimulation, most of the PMBCs maintained their integrity; however, in such preparations cytoplasmic fragments were also evident. PHA-stimulated PMBCs were usually larger, with roundish nuclei often showing a crown of dense chromatin near the nuclear envelope. The cytoplasm contained many free ribosomes and the smooth endoplasmic reticulum was organized in cisternae and post-Golgian vesicles. Smaller (200–250 nm) vesicles showed an electron-transparent content, while the larger (300–500 nm) ones had an electron-dense, flocculent core. Larger vesicles were more often localized in the periphery of the cytoplasm. The

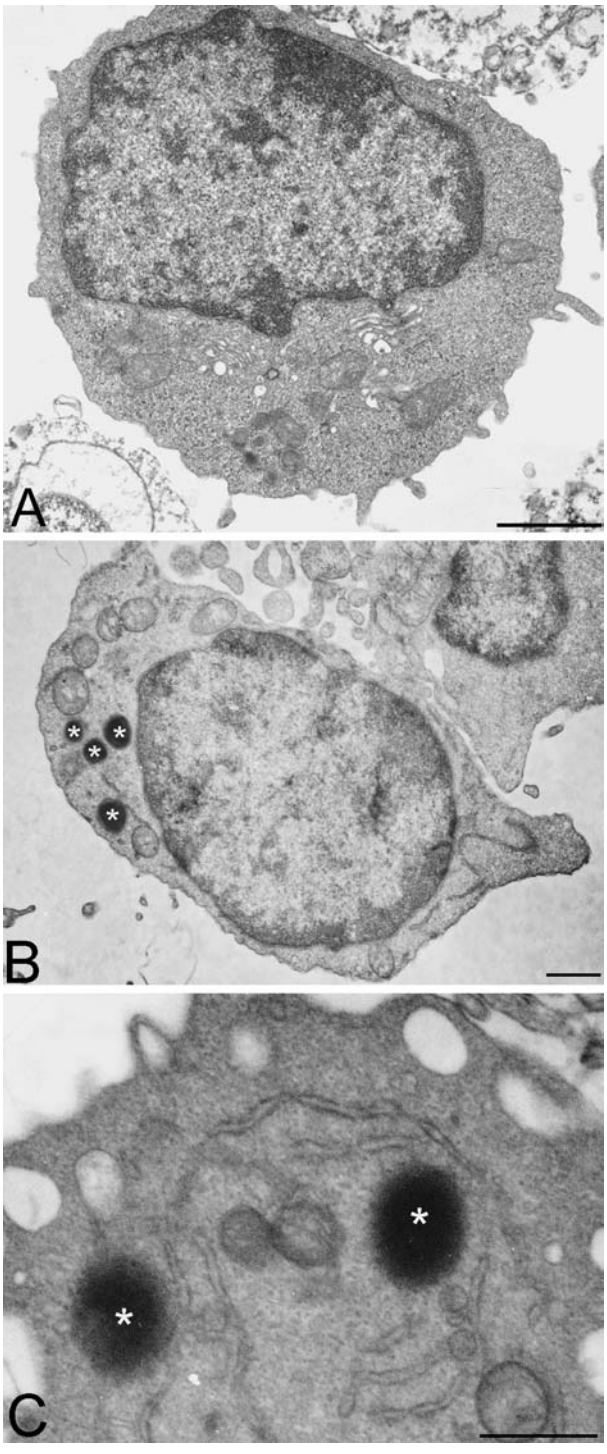

Fig. 4A–C Electron micrographs of human PBMCs after stimulation with PHA. **A** Cell stained with lead citrate and uranyl acetate. **B** Unstained cell labelled for TH. In TH-positive cells stimulated with PHA, specific immunoreactivity for TH was almost exclusively localized to large, electron-dense cytoplasmic granules (*asterisks*). **C** Detail of TH-positive cytoplasmic granules (*asterisks*). TH-positive granules could occasionally be observed near the plasma membrane, which was, however, always free from immunoreaction. *Scale bars* 2 μm **(A)**, 1 μm **(B)**, 500 nm **(C)**

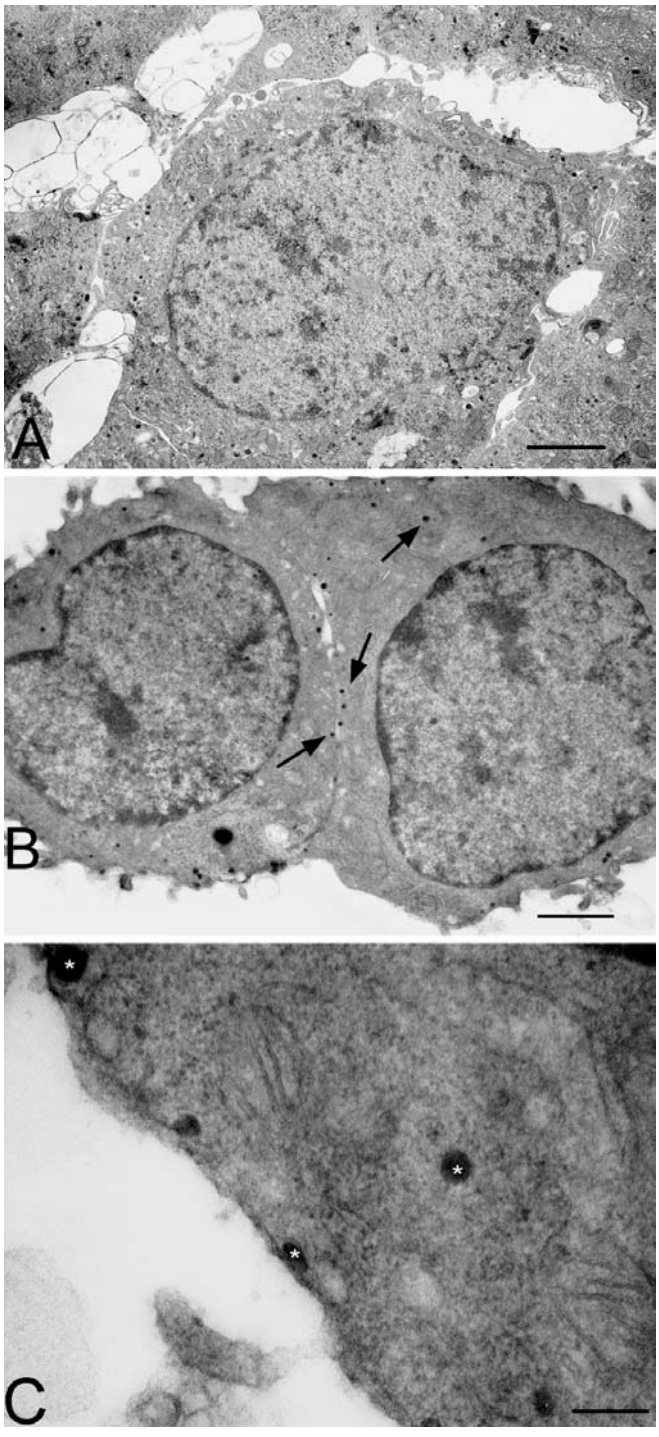

Fig. 5A–C Electron micrographs of PC12 cells. **A** Cell stained with lead citrate and uranyl acetate. **B** Unstained cell labelled for TH. TH-positive granules (*arrows*) quite homogeneous in size were visible at the periphery of the cytoplasm. **C** Detail of TH-positive granules (*asterisks*). TH-specific immunoreactivity was localized to cytoplasmic granules near the plasma membrane, which was completely free from TH-specific labelling. *Scale bars* 1 μm **(A, B)**, 100 nm **(C)**

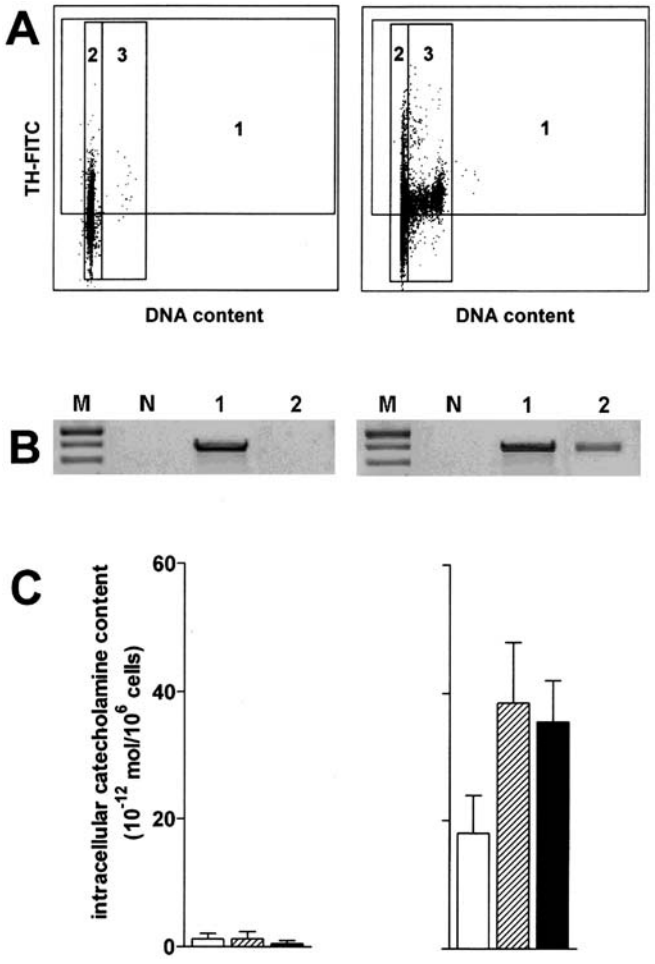

Fig. 6 Unstimulated human PBMCs (*left*) were about 50% positive for TH **(A)**, but did not express TH mRNA **(B)** and contained low levels of catecholamines **(C)**. On the contrary, after 48 h stimulation with PHA, 10 μg/ml (*right*) positivity for TH increased in relationship with the cell cycle **(A)**, TH mRNA could be detected **(B)** and intracellular catecholamines increased about 20- to 40-fold **(C)**. **A** *1* TH-positive cells, *2* G0/G1 cells, *3* S+G2/M cells. Data from one representative of ten experiments. **B** *M* molecular weight markers, *N* negative control (no RNA), *lane 1* PC12 cells, *lane 2* human PBMCs. Data from one representative of six experiments. **C** *empty columns* dopamine, *hatched columns* norepinephrine, *filled columns* epinephrine. Data are means ± SD of six experiments

mitochondria usually presented many thin cristae (Fig. 4A). In PHA-stimulated PBMCs, specific immunoreactivity for TH was almost completely localized to larger (300–500 nm), electron-dense cytoplasmic granules which could be occasionally observed also in close proximity to the plasma membrane (Fig. 4B). At variance with unstimulated cells, after PHA stimulation plasma membranes and periplasmalemmal smooth reticulum cisternae showed no detectable TH immunoreactivity (Fig. 4C).

For comparison, PC12 cells contained round, oval or irregularly shaped granules with variable electron densities. Granules were about 100–200 nm in size with a very electron-dense core and were usually localized at

Table 1 Flow cytometry analysis of TH expression in human PBMCs. Values are means ± SD of ten observations. For further details, see text

TH expression	Unstimulated	PHA-stimulated			*P* (G0/G1 vs S+G2/M)
		Total	G0/G1	S+G2/M	
Fluorescence ratio	2.626±1.032	3.930±2.524	3.531±2.568	3.933±3.080	NS
Positive cells (%)	46.2±33.7	50.2±25.6	43.6±27.6	82.7±15.6	<0.005

the periphery of the cytoplasm, immediately under the plasma membrane (Fig. 5A). Strong and uniform TH immunoreactivity was seen in these granules (Fig. 5B). These granules were sometimes observed in close contact with the plasma membrane, which was always negative for TH immunoreactivity (Fig. 5C).

Flow cytometry analysis of TH expression

MFI in TH-labelled PBMCs was 0.468±0.210 (n=14) and was significantly higher than in unlabelled PBMCs (0.176±0.030, n=14; P<0.0005 vs TH-labelled cells by two-tailed Student's t-test for paired data). Specific labelling for TH in both unstimulated and PHA-stimulated PBMCs was evident in about half of the cells, with a high degree of variability among different samples, from less than 20% to more than 90% TH-positive cells. PHA-stimulated PBMCs, however, showed increased TH labelling (Fig. 6A) mainly due to the high frequency of TH-positive cells in the S+G2/M phases of the cell cycle (Table 1).

TH mRNA expression and intracellular catecholamine levels

Unstimulated PBMCs contained no detectable levels of TH mRNA, and the catecholamines dopamine, norepinephrine and epinephrine were present in low amounts, in agreement with previous studies showing that in freshly isolated PBMCs catecholamine levels are in the range of $0.1–1\times10^{-12}$ mol/10^6 cells, possibly due also to uptake of extracellular (plasma) catecholamines (Marino et al. 1999). As expected (Cosentino et al. 2002), PHA stimulation induced the expression of TH mRNA, followed by a huge enhancement of intracellular catecholamines, which increased by 20- to 40-fold (Fig. 6B, C).

Discussion

The results of the present study provide for the first time a direct description of the ultrastructural localization of TH in human PBMCs. Using immunocytochemistry coupled to fluorescence and electron microscopy and to flow cytometry we have shown that TH is expressed in both unstimulated and PHA-stimulated PBMCs. However, in unstimulated cells TH is mainly associated with the plasma membrane, while in stimulated cells it is localized in large, dense cytoplasmic granules associated with the Golgi apparatus, and the plasma membrane shows no detectable TH immunoreactivity.

The immunoelectron-microscopic technique adopted in this study uses a mouse anti-TH monoclonal antibody and has been performed following the steps described by Oomori et al. (1989) for TH staining in PC12 cells. Our control observations performed on PC12 cells showed results which are perfectly superimposable on the ones by Oomori and coworkers. Moreover, the absence of diaminobenzidine deposition in both negative and isotypic control specimens indicates the absence of cross-reactivity with non-specific binding sites on PBMCs or PC12 cells, further supporting the specificity of TH immunoreactivity in labelled samples.

Recently, TH expression in human PBMCs was demonstrated by use of an immunofluorescence technique (Amenta et al. 2001). However, the subcellular localization of the enzyme can be revealed only by immunoelectron-microscopic investigation. Indeed, although in our experiments light-microscopic examination suggested a different arrangement of TH in PBMCs before and after stimulation with PHA (inasmuch as specific fluorescence was restricted to the plasma membrane of unstimulated cells, but was diffused to the cytoplasm and possibly localized to small granules in stimulated cells), such differences could be confirmed and described in detail only by ultrastructural analysis.

When examined by immunoelectron microscopy, unstimulated PBMCs showed an evident electron-dense positivity localized to the plasma membrane and to the underlying very thin cisternae pertaining to the smooth reticulum. Such membrane-localized positivity completely disappeared in PHA-stimulated PBMCs. In these conditions, specific immunoreactivity for TH was localized exclusively to large cytoplasmic granules. Comparison of TH-positive granules in PHA-stimulated PBMCs and in PC12 cells shows some remarkable similarities, inasmuch as in both cell types the granules lie prevalently at the periphery of the cell, sometimes in direct contact with the plasma membrane. Nevertheless, in PBMCs the diameter of TH-positive granules ranges from 300 to 500 nm while in PC12 cells it lies between 100 and 200 nm. Oomori et al. (1989) suggested that in rat adrenal chromaffin and PC12 cells TH may co-localize with catecholamines in the same granules, a hypothesis which could provide the structural basis for the well-documented catecholamine-dependent regulation of TH activity. We have no direct evidence that TH-positive granules in PBMCs also contain catecholamines. However, in line with previous recent observations (Cosentino et al.

2002), our results clearly show that PHA stimulation induced a 20- to 40-fold enhancement of intracellular catecholamines, together with the expression of TH mRNA. Intracellular catecholamine content peaked at 48 h and did not increase further, while extracellular catecholamine levels in the culture medium were not affected by PHA, suggesting that catecholamines were stored in the cells and did not undergo immediate release. As discussed above (see "Introduction"), in catecholamine-producing cells modifications of TH activity are accompanied by intracellular redistribution of the enzyme. Our observations now offer direct support for the hypothesis that even in immune cells TH may exist in different functional states in relationship with its ultrastructural localization. As a working hypothesis, we propose that during PBMC activation TH is upregulated at different levels, including gene transcription and enzymatic activity, thus resulting in increased synthesis of catecholamines. Newly synthesized catecholamines in turn colocalize with TH in cytoplasmic granules, where catecholamine accumulation might eventually result in inhibition of TH activity.

Provided that TH-positive granules represent storage sites for catecholamines, the question arises whether morphological differences between PBMCs and PC12 cells may imply the existence of distinct mechanisms regulating catecholamine release. Catecholamine release from neurons and neuroendocrine cells occurs through both exocytotic, Ca^{2+}-dependent and non-exocytotic, transporter-mediated mechanisms. In PC12 cells, regulated release of catecholamines classically involves rapid, local rises of intracellular Ca^{2+} concentrations due to Ca^{2+} influx through voltage-gated Ca^{2+} channels, and TH-positive granules near the plasma membrane may therefore represent a readily releasable pool in response to cell depolarization. TH-positive granules in PBMCs, however, differ from those in PC12 cells as regards both size and localization. Differences in the activity of the enzyme and in the conditions of release of granular contents cannot therefore be ruled out. In any case, the loss of TH positivity of the plasma membrane in PHA-stimulated PBMCs needs further investigation to assess its functional implications. In preliminary experiments we have found that in PBMCs Ca^{2+} ionophores such as ionomycin and A23187 do not induce catecholamine release (unpublished observations). It was previously shown that unstimulated PBMCs release catecholamines in the presence of reserpine (Marino et al. 1999; Cosentino et al. 2000), supporting the existence and the functional relevance of monoamine transporters. Indeed, dopamine plasma membrane and vesicular transporters have been recently identified in human peripheral blood lymphocytes (Amenta et al. 2001).

TH was found to be expressed in only 46–50% of total PBMCs, and stimulation with PHA did not significantly affect the proportion of TH-positive cells. Such a distribution was evident even by light- and electron-microscopic examination and was unequivocally confirmed by flow cytometry analysis. This finding suggests that TH expression (and the subsequent ability to produce catecholamines) may be restricted to one or more cell subset(s), in agreement with previous studies showing that different leucocyte subpopulations and haematopoietic cell lines may contain catecholamines to a different extent (Bergquist et al. 1998; Cosentino et al. 2000). Identification of the specific type of TH-positive cells was beyond the purpose of this study. PBMCs are indeed heterogeneous in nature as they contain both T- and B-lymphocytes as well as a variable proportion of monocytes. Although in our conditions lymphocytes and monocytes were always about 80% and 16% respectively, the various experimental procedures, including isolation, culture, stimulation and processing, profoundly affected their ultrastructural morphology. Microscopic examination therefore did not allow the unequivocal identification of the cells. In this regard, we have currently undertaken specific experiments by use of immunomagnetic cell sorting, as it is possible that differences in TH expression and/or catecholamine levels may imply differences in the functional role of catecholamines in distinct cell subsets.

Interestingly, in PHA-stimulated PBMCs there was a clear relationship between TH expression and cell proliferation, TH being expressed with greater frequency in cells in the S-G2/M phases of the cell cycle. It was recently shown that in immune cell lines TH expression correlated with cell growth (Tsao et al. 1998), and in mouse bone marrow norepinephrine content was positively associated with the proportion of cells in the S-G2/M phases of the cell cycle (Maestroni et al. 1998). These observations strengthen the hypothesis that catecholamines may play a role in the functional modulation of proliferating immune cells.

In conclusion, we have shown for the first time in human PBMCs the ultrastructural localization of TH, the rate-limiting enzyme in the synthesis of catecholamines. The rearrangements occurring in stimulated cells strongly support the functional relevance of catecholaminergic mechanisms during cell activation. Studies are in progress to assess the role of immune-cell-derived catecholamines in the regulation of cell-cell interactions in the immune system and in the cross-talk between the immune system and the nervous system both in health and in disease conditions.

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