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Melatonin biosynthesis and metabolism in peripheral blood mononuclear leucocytes

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Cultured human peripheral blood mononuclear leucocytes (PBML) were able to synthesize indoleamines, including melatonin, and were also able to convert melatonin taken up from the incubation medium into *N*-acetyl-5-hydroxytryptamine (NAHT) and 5-hydroxytryptamine (5-HT). These compounds were analysed by h.p.l.c., and melatonin was additionally characterized by two-dimensional t.l.c., mass spectrometry and radioimmunoassay. Only hydroxyindoles were detected by h.p.l.c. in unstimulated PBML culture. Sustained stimulation by melatonin or interferon-γ (IFN-γ) increased markedly the basal production of 5-HT. IFN-γ- or 5-HT-stimulated (but not resting) cells produced NAHT and melatonin. Furthermore, the addition of melatonin to the culture medium strongly enhanced NAHT and 5-HT production without affecting tryptophan hydroxylation, suggesting the possibility of direct or indirect transformation of melatonin into NAHT and 5-HT.

INTRODUCTION

Several studies have suggested that indoleamines are involved in modulation of the immune system [1–3]. 5-Hydroxytryptamine (5-HT; serotonin) and melatonin have been shown to regulate natural killer cell cytotoxicity [2,4,5], to alter antibody production in mice [6–8] and to inhibit *in vitro* mitogen-stimulated lymphoproliferation and interferon-γ (IFN-γ) production by human T lymphocytes [9].

In previous work it was demonstrated that lymphocytes and macrophages can metabolize tryptophan to 5-HT and melatonin, a process that is markedly stimulated by IFN-γ [10]. Uptake of both tryptophan and 5-HT was observed [1,10]. IFN-γ appears to enhance both tryptophan uptake [10] and degradation via the kynurenine catabolic pathway [11], as well as increasing the biosynthesis of pterins in peripheral blood mononuclear leucocytes (PBML) [12]. Tetrahydrobiopterin is a cofactor for tryptophan hydroxylase, the enzyme that regulates the biosynthesis of 5-HT, which in turn can be metabolized into melatonin. When IFN-γ and 5-HT are added together to a lymphocyte culture, increased production of *N*-acetyl-5-hydroxytryptamine (NAHT) and melatonin can be observed [10]. IFN-γ enhances melatonin synthesis in PBML by decreasing oxidative deamination of 5-HT and switching the biosynthetic pathway towards melatonin production [10].

The present paper confirms the occurrence of melatonin synthesis in PBML and provides direct evidence that PBML have the capacity to metabolize not only 5-HT into melatonin but also melatonin into 5-HT, depending upon the ratio of available substrates present in the cells.

MATERIALS AND METHODS

Cell cultures

PBML (75% lymphocytes/25% monocytes) were obtained by density-gradient separation on a Ficoll–Hypaque mixture (Pharmacia Fine Chemicals, Uppsala, Sweden) by a standard procedure [13]. Cells were cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum at 37 °C in a humid atmosphere of 5% CO_2 and 95% air, as has been previously described [10].

Stimulation by indoleamines and IFN-γ

Cells were plated (96-well plates) and incubated for 30 min at 37 °C. Then the different wells were treated with 1 mM-5-HT, 1 mM-melatonin or highly purified natural human IFN-γ (900 units/ml) (kindly provided by BioSidus S.A., Buenos Aires, Argentina). The IFN-γ control was performed by preincubating IFN-γ with anti-(human IFN-γ) antiserum (kindly provided by Dr. E. Falcoff, Institut Curie, Paris, France) for 1 h at 37 °C. Incubation and further experimental procedures were performed in the dark to avoid photosensitive drug degradation.

Determination of tryptophan hydroxylase

PBML were incubated for up to 48 h in RPMI-1640 culture medium containing 50 μM-tryptophan to which L-[5-^{3}H]tryptophan (NET-782; New England Nuclear, Boston, MA, U.S.A.; sp. radioactivity 12.3 Ci/mmol) was added at a final concentration of 2.5 μCi/ml. Since Ficoll has been described as a tryptophan hydroxylase inhibitor [14], in some assays cells were preincubated for 23 or 47 h before a 1 h pulse of radioactive tryptophan in the absence or the presence of the compounds mentioned in the section above. Control wells received culture medium without PBML to determine non-specific tryptophan degradation during the experiment. The amount of tryptophan entering the 5-hydroxyindole pathway in the cultured PBML was determined by measuring the amount of ^3H_2O formed following acidification of the media and homogenates by a modification of the method of Bensinger *et al.* [15]. The ^3H_2O was separated from the substrate and other hydroxylated products by h.p.l.c. μBondapack C_{18} elution using 10 mM-triethylamine adjusted to pH 3 with phosphoric acid. Fractions (1 ml) were collected in a

Abbreviations used: 5-HT, 5-hydroxytryptamine (serotonin); NAHT, *N*-acetyl-5HT; IFN-γ, interferon-γ; PBML, peripheral blood mononuclear leucocytes; 5-HTP, 5-hydroxytryptophan; 5-HIAA, 5-hydroxyindole-3-acetic acid.

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single scintillation vial and counted for radioactivity by liquid scintillation spectrometry in a Packard Tri-Carb liquid scintillation spectrometer (Rockville, MD, U.S.A.) at 30% counting efficiency.

Further proof of peak identity was obtained by comparing the total amount of radioactivity in the first h.p.l.c. peak with the effluent from small columns of Dowex 50W-X8, hydrogen form (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Determination of hydroxyindoles and methoxyindoles

After 2, 12, 16, 24 or 48 h incubations in RPMI-1640 medium containing 50 μM-L-tryptophan in the absence or the presence of the compounds mentioned previously, indole metabolites were determined by h.p.l.c. as described by Finocchiaro *et al.* [16].

Statistical analysis

Results were expressed as means ± S.E.M. ($n = 4$) of the amounts liberated into the medium plus tissue content(s) of the assayed substances. Analysis of variance and Tukey's test for individual mean differences were performed.

Preparation of human PBML extract

After 48 h incubation in RPMI-1640 culture medium containing 100 μM-[^{3}H]5-HT (5-[1,2-^{3}H(n)]NET 498) and 900 units of IFN-γ/ml, medium and homogenates were adjusted to pH 10 with sodium borate buffer and the radioactivity was extracted with chloroform according to the method of Launay *et al.* [17]. The alkaline-chloroform extracts were pooled and evaporated to dryness and then subjected to one-dimensional t.l.c., in parallel with a melatonin standard as described [17,18]. Spots identified under u.v. light having an R_F equivalent to that of the melatonin standard were scraped off the t.l.c. plate, extracted from the silica gel using ethanol, pooled and evaporated under a nitrogen stream to a final volume of 1 ml. An aliquot (50 μl) of this ethanol solution (fraction M) was then counted for radioactivity as previously described.

Two-dimensional t.l.c.

Two-dimensional t.l.c. was performed on 30 μl portions of fraction M containing 10 μg of each indole standard as described [17,18]. After localization under u.v. light, the spots corresponding to the different standards were scraped off and the radioactivity was counted as previously described.

Mass spectrometry

After direct introduction of the sample without derivatization, mass spectrometry was performed by electron impact on authentic melatonin and on dried fraction M using an AEI spectrometer type 50, as previously described [17,18]. Mass spectrometer conditions were: ionization chamber temperature, 180 °C; electron energy, 70 eV.

Radioimmunoassay

Aliquots of a solution of melatonin standard and of fraction M were measured by radioimmunoassay as previously described [17–19]. The standard curve was established using (in duplicate) six different concentrations of unlabelled melatonin (from 272 to 8.5 fmol in 2-fold dilution steps).

RESULTS

Tryptophan hydroxylase activity

The first metabolic step in the synthesis of indoleamines is the hydroxylation of tryptophan. During a 48 h incubation with 50 μM-tryptophan, 5×10^5 PBML hydroxylated 250 pmol of tryptophan. The amount of this amino acid entering the 5-hydroxyindole pathway in the cultured PBML was linear over this 48 h period (Fig. 1*a*).

The effects of a 23 h preincubation with 5-HT, melatonin or IFN-γ on tryptophan hydroxylation after a 1 h pulse were examined (Fig. 1*b*). Whereas 1 mM-5-HT inhibited hydroxylation by 50% ($P < 0.05$), 1 mM-melatonin did not significantly affect it, and 900 units of IFN-γ/ml doubled the rate of enzyme activity ($P < 0.01$). Similar values were obtained when the tryptophan hydroxylase activity was measured between 23 and 24 h or between 47 and 48 h (results not shown). This result substantiates the linearity of tryptophan hydroxylation over the incubation period.

Methoxyindole and hydroxyindole production by PBML

Fig. 2 shows the time course of total 5-HT, NAHT and melatonin production by PBML under different conditions. While PBML by themselves synthesized detectable amounts of 5-HT, the addition of melatonin or IFN-γ increased, by up to 10- and 13-fold respectively, the basal production of this indoleamine at 24 h ($P < 0.01$) (Fig. 2*a*).

NAHT was only detected when cells were in the presence of 5-HT or IFN-γ, and it gradually increased with the incubation time (Fig. 2*b*). Melatonin added to the PBML culture markedly enhanced the production of NAHT at early incubation times, reaching after 16 h a maximum of a 100-fold increase over the values obtained with other treatments ($P < 0.01$).

IFN-γ- or 5-HT-stimulated (but not resting) cells produced

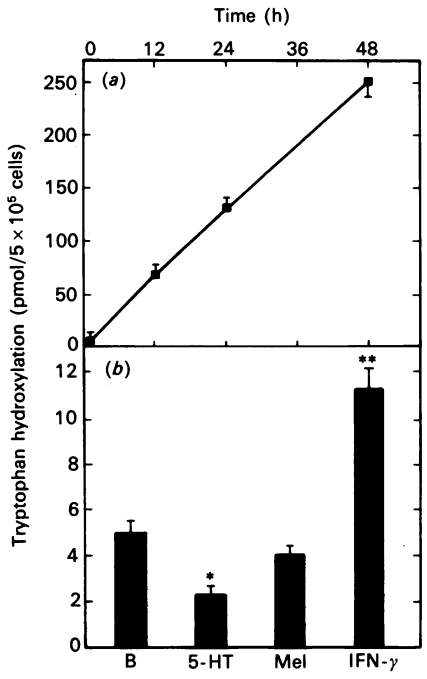

Fig. 1. Tryptophan hydroxylation by PBML

(*a*) Time course. Cells were incubated in RPMI 1640 containing 50 μM-5-[^{3}H]tryptophan as described in the Materials and methods section. Each point represents the mean ± S.E.M. of four determinations of the amount of 5-[^{3}H]tryptophan hydroxylated by 5×10^5 cells. (*b*) Effects of 5-HT, melatonin (Mel) and IFN-γ on tryptophan hydroxylation. Cells were preincubated in RPMI 1640 (50 μM-L-tryptophan) in the absence (basal; B), or presence of the above-mentioned compounds (1 mM-indole or 900 units of IFN-γ/ml) for 23 h. Each point represents the mean ± S.E.M. of four determinations of the amount of 5-[^{3}H]tryptophan hydroxylated in 1 h by 5×10^5 cells determined as described in the Materials and methods section. *$P < 0.05$ and **$P < 0.01$ compared with basal levels.

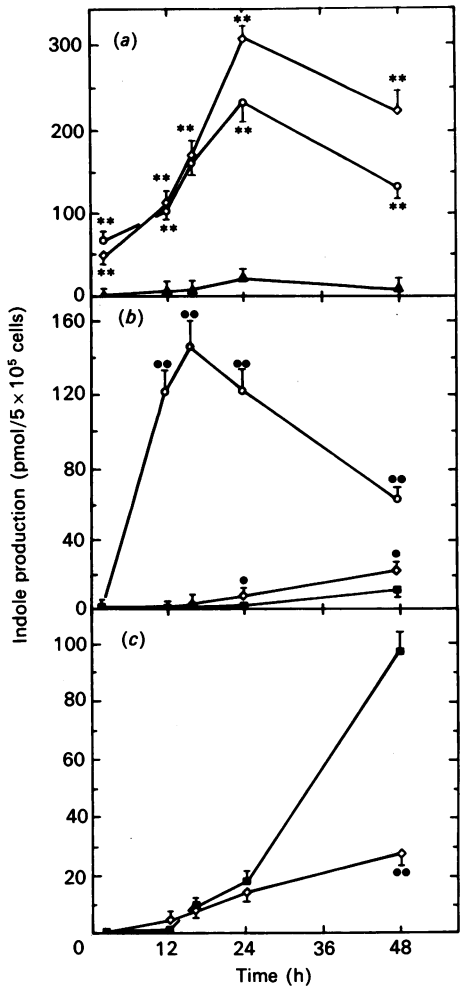

Fig. 2. Effects of 5-HT (■), melatonin (○) and INF-γ (◇) on PBML indole production

Cells were incubated in RPMI 1640 (50 μM-L-tryptophan) in the absence (basal, ▲) or the presence of the above-mentioned compounds (1 mM-indole or 900 units of IFN-γ/ml) for the indicated times. Indole metabolites determined by h.p.l.c. as described in the Materials and methods section. Concentrations of (*a*) 5-HT, (*b*) NAHT and (*c*) melatonin are expressed as means ± S.E.M., $n = 4$. $^{*}P < 0.05$, $^{**}P < 0.01$ with respect to basal levels; $^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.01$ with respect to 5-HT-stimulated levels.

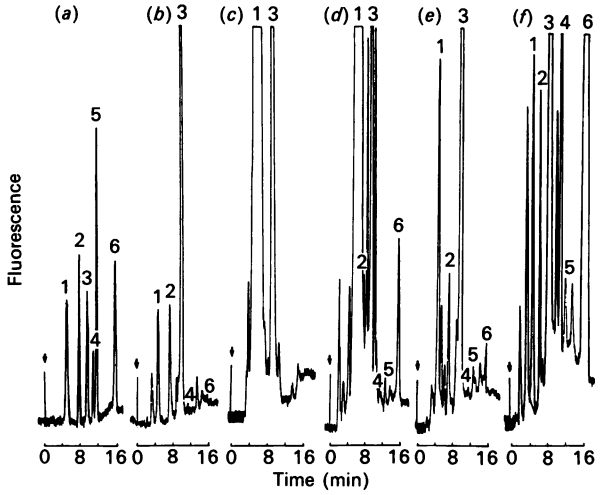

Fig. 3. H.p.l.c. determination of methoxyindoles and hydroxyindoles in the culture medium of PBML

Cells were incubated in RPMI 1640 (50 μM-L-tryptophan) without additions for 48 h (*b*), with 1 mM-5-HT for 2 h (*c*) or 48 h (*d*), with 900 units of IFN-γ/ml for 48 h (*e*) or with 1 mM-melatonin for 48 h (*f*). Indole metabolites were detected by native fluorescence with an excitation wavelength of 254 nm and an emission filter of 360 nm. Chromatographic conditions and peak identity are described in the Materials and methods section. The time of injection is indicated with an arrow. (*a*) Standards: 1, 5-HT; 2, 5-HTP; 3, tryptophan; 4, 5-HIAA; 5, NAHT; 6, melatonin.

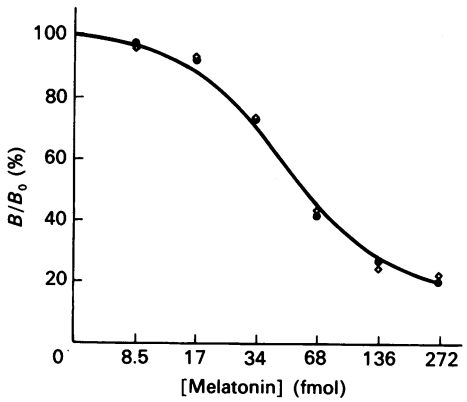

Fig. 4. Displacement of [^{3}H]melatonin from a specific anti-melatonin immunoserum by fraction M (◇) and melatonin standard (●)

A Scatchard-type analysis was done with three series of experimental points. Inhibition of [^{3}H]melatonin fixation on the immunoserum is indicated: B, melatonin bound at given concentration; B_0, melatonin bound in the absence of inhibitors.

melatonin (Fig. 2*c*). Whereas IFN-γ slowly increased melatonin production, 5-HT markedly stimulated it at 48 h, being 4-fold more effective than the peptide ($P < 0.01$).

When IFN-γ was preincubated with anti-(human IFN-γ) antiserum, all of the IFN-γ effects described were eliminated (results not shown).

Evidence for melatonin biosynthesis in PBML

H.p.l.c. Fig. 3 shows a chromatogram of the determination of tryptophan, 5-hydroxytryptophan (5-HTP), 5-HT, NAHT, melatonin and 5-hydroxyindole-3-acetic acid (5-HIAA) produced by PBML under different conditions, run with the appropriate standards. In a 48 h unstimulated PBML culture only hydroxyindoles (5-HTP, 5-HT, 5-HIAA) were detected (Fig. 3*b*). The addition of 1 mM-5-HT to the incubation medium yielded a peak that co-eluted with melatonin after 48 h of stimulation (Fig. 3*d*), but not after 2 h (Fig. 3*c*), indicating that this peak was not a 5-HT impurity but a product of PBML 5-HT metabolism.

IFN-γ (900 units/ml) specifically increased 5-HT production but also had a small stimulatory effect on the other indoles (Fig. 3*e*). The presence of 1 mM-melatonin in the incubation medium markedly enhanced the 5-HTP, 5-HT, 5-HIAA and NAHT peaks (Fig. 3*f*).

T.l.c. When the alkaline/chloroform extract of PBML (see the Materials and methods section) was subjected to one-dimensional t.l.c. in parallel with a melatonin standard, a labelled compound was found with the same R_F as melatonin. This compound, extracted with ethanol (fraction M), was then subjected to two-dimensional t.l.c., mass spectrometry and r.i.a.

Two-dimensional t.l.c. yielded only one radioactive spot co-migrating with melatonin (results not shown).

Mass spectrometry

The electron-impact mass spectrum of the fraction M compound was compared with that of authentic melatonin. Both produced similar fragmentation patterns, including a molecular peak (m/z 232 M^+) and four other ions (m/z 173, 160, 145, 117) with close relative intensities. The fragment m/z 149, evident only in the mass spectrum of fraction M, is probably due to t.l.c. impurities.

Radioimmunoassay

As shown in Fig. 4, the fraction M compound competed with [^{3}H]melatonin, binding to its specific immunoserum and showing the same concentration-dependence as that of authentic melatonin. It is worth noting that the radioactivity of fraction M, detected after 1:10000 or higher fold dilutions, was equivalent to the background levels of the scintillation counter.

DISCUSSION

In an attempt to clarify some aspects of the regulation of indole metabolism, we studied the effects of pharmacological manipulations on tryptophan hydroxylation and on the production of indoleamines by cultured PBML. With this approach it was possible to detect changes in PBML indoleamine synthesis which were related to changes in substrate levels or uptake, cofactor availability, hormonal influences and substrate feedback regulation.

The tryptophan hydroxylase activity in cultured PBML was linear during a 48 h culture period (Fig. 1*a*), as occurs in cultured pineal gland [15]. The cells seem to have the capacity to regenerate reduced pteridine cofactor [12] and to efficiently use iron stores.

The presence of external 5-HT enhanced the production of NAHT and melatonin (Figs. 2*b* and 2*c*), but decreased tryptophan hydroxylation by 50% (Fig. 1*b*) without modifying basal tryptophan uptake (results not shown). These results agree with previous reports [10], and suggest that elevated levels of 5-HT may exert a negative feedback control on tryptophan hydroxylase activity, switching the metabolic pathway towards melatonin production.

Although melatonin stimulation of PBML led to a significant increase in 5-HT levels, the most marked effect was elicited on NAHT, whose production increased dramatically (Fig. 2*b*). Melatonin stimulated NAHT and 5-HT production (Figs. 2*b* and 2*a*) without affecting tryptophan uptake (results not shown) or hydroxylation (Fig. 1*b*). It is therefore possible that melatonin, at least at pharmacological doses, could be demethylated and deacetylated, and thus transformed into NAHT and 5-HT. Synthesis of 5-methoxytryptamine has already been described in the rat, via deacetylation of melatonin by the enzyme aryl acylamidase [20]. It is interesting to note that exogenous melatonin, when given to rats, can be metabolized to NAHT, as determined by gas chromatography–mass spectrometry [21], and induces an increase in pineal 5-HT content [22]. However, in our study, pharmacological doses of melatonin were used. Whether NAHT and 5-HT are significant metabolites of melatonin under physiological conditions remains to be determined.

It is worth noting that the NAHT production elicited by melatonin was markedly higher than that elicited by 5-HT. The production of NAHT may depend on the differential regulation of the enzymes involved in its biosynthesis. The *N*-acetyltransferase activity at 1 mM-5-HT seemed markedly lower than the demethylation of millimolar levels of melatonin. These results suggest an inhibition of PBML *N*-acetyltransferase activity by high concentrations of 5-HT, as occurs in the pineal gland [23,24].

The addition of IFN-γ to the culture medium markedly enhanced tryptophan hydroxylation (Fig. 1*b*) and the basal production of 5-HT, NAHT and melatonin (Fig. 2). This was not surprising, since the peptide is known to stimulate tryptophan uptake and increase its intracellular concentration [10], as well as stimulating the biosynthesis of pterins [12] and the production of 5-HT [10] in PBML.

In PBML, IFN-γ is a potent inducer of indoleamine dioxygenase activity, which catalyses the oxygenative ring cleavage of tryptophan and various indoleamine derivatives such as 5-HTP, tryptamine, 5-HT [25,26] and melatonin [27], resulting in the depletion of tryptophan levels [25,26]. From preliminary experiments, IFN-γ appeared to enhance both tryptophan and 5-HT degradation via the kynurenine catabolic pathway. It remains to be established whether the PBML kynurenine catabolites observed *in vitro* and *in vivo* after IFN-γ induction are also derived from melatonin degradation. The effect of IFN-γ on tryptophan availability for the various metabolic routes exhibited by PBML suggested an important mechanism to regulate the metabolism of the least available amino acid, L-tryptophan. The growth, DNA synthesis and protein synthesis of the cells [25], as well as the indolic pathway [10], are markedly influenced by the concentration of L-tryptophan. In addition, by modulation of the indoleamine dioxygenase activity, IFN-γ can regulate the synthesis and turnover of 5-HT and melatonin [10], which in turn inhibit IFN-γ synthesis [9]. All these facts support the existence of a fine immunoregulatory circuit as part of the molecular mechanisms of anti-viral, anti-proliferative and immunomodulatory actions of IFN-γ [25].

Finally, by means of multiple biochemical assays, we confirmed the identity of the PBML-synthesized melatonin. A first indication was obtained by h.p.l.c. The addition of 1 mM-5-HT to the incubation medium yielded a peak that co-eluted with melatonin after 48 h of stimulation but not after 2 h, indicating that this peak was not a 5-HT impurity but a product of PBML 5-HT metabolism. A second indication of melatonin identity was obtained by t.l.c. Indeed, the alkaline/chloroform extract of PBML incubated with 5-HT [^{3}H] contained a labelled compound which migrated on t.l.c. as does authentic melatonin (results not shown). After removal of the spot suspected to be melatonin from the silica gel, its identity was confirmed by two-dimensional t.l.c. (results not shown), its mass fragmentation pattern, and the displacement curve of [^{3}H]melatonin from its specific immunoserum (Fig. 4).

The present study gives clear evidence that PBML have the capacity to metabolize not only 5-HT into melatonin but also melatonin taken up from the incubation medium into NAHT and 5-HT, depending upon the ratio of available substrates present in the cells. We can conclude that PBML contain all of the enzymic machinery involved in the tryptophan indole pathway.

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