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Tryptophan consumption and indoleamines production by peritoneal cavity macrophages

E. Martins Jr.,* A. C. F. Ferreira,* A. L. Skorupa,† S. C. Afeche,‡ J. Cipolla-Neto,† and L. F. B. P. Costa Rosa*,[1]

*Departments of *Histology and Embryology and †Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, Brazil; and ‡Butantan Institute, São Paulo, Brazil*

Abstract: Melatonin has been shown to regulate several immune functions, and some authors showed that leukocytes are also able to produce the indolamine. In fact, it seems to take part in some immunoregulatory axis, including that related to interferon (IFN) production. So, we evaluated the rate of tryptophan consumption and melatonin and serotonin production in peritoneal cavity-isolated macrophages and the effect of IFN-α and -γ, lipopolysaccharide (LPS), and phorbol myristate acetate (PMA) on such parameters. Our results indicate that macrophages obtained from the peritoneal cavity of normal rats when incubated with tryptophan show an increase in arylalkylamine N-acetyltransferase activity that corresponds to an increased melatonin production, as determined in the incubation medium. This process is regulated by IFN-α and -γ, PMA, LPS, and the serum from tumor-bearing rats, opening the possibility of speculation about different immunoregulatory loops acting through the balance of melatonin/serotonin production by such cells. *J. Leukoc. Biol.* 75: 000–000; 2004.

Key Words: *melatonin production · serotonin · interferon*

INTRODUCTION

Several experiments have been performed recently to identify the mechanism(s) responsible for the bidirectional communication between the neuroendocrine and immune systems [1]. The link between these systems depends on several immune factors, hormones, and common receptors [2–4]. Among these, a possible candidate, in whose favor a large scope of evidence has been put forward [5–8], is the neurohormone melatonin (*N*-acetyl-5-methoxytryptamine).

Melatonin has been shown to regulate several immune functions, such as natural killer (NK) cell cytotoxicity [9, 10], antibody production [11–13], lymphocyte proliferation, interferon-γ (IFN-γ) production [14], and T helper cell type 2 function [5]. Some experiments have also demonstrated an indirect effect of melatonin on the immune system through the endogenous opioid system, which is able to control macrophage and lymphocyte function [15, 16], or through an effect on bone marrow. Conversely, Finocchiaro and colleagues [7] showed that peripheral blood mononuclear leukocytes (PBML) have the capacity to metabolize serotonin 5-hydroxytryptamine (5-HT) to melatonin, opening the possibility to the existence of a two-way pathway in the neuroendocrine system. In fact, concerning IFN-γ, the indolamines seem to be part of an immunoregulatory circuit, as IFN-γ stimulates the production of serotonin and melatonin by macrophages and lymphocytes, whereas these indolamines inhibit the synthesis of IFN-γ [17, 18].

Therefore, to better understand the mechanisms involved in a local production of melatonin by immunological cells, we evaluated the rate of tryptophan consumption and melatonin and serotonin production in peritoneal cavity-isolated macrophages and the effect of IFN-α and -γ, lipopolysaccharide (LPS), and phorbol myristate acetate (PMA) on such parameters.

MATERIALS AND METHODS

Animals

Weaned, male, albino Wistar rats, weighing 150–200 g (2–4 months of age, n=48), were kept under a 12h:12h light-dark cycle (lights on at 7:00 AM) in a temperature- and humidity-controlled (21±2°C, 70%) room with water and food ad libitum.

All procedures were approved by the Institute of Biomedical Sciences (University of São Paulo, Ethical Committee for Animal Research, Brazil; CEEA) and are in agreement with the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation (COBEA).

Reagents

Reagents were obtained from Sigma Aldrich (Dorset, UK/St. Louis, MO), Boehringer Mannheim (UK), Gibco (supplied by Bio-Sciences, Dublin, Ireland), and Quimibras Industrial Brasileira SA (Rio de Janeiro, Brazil). Radiochemicals were obtained from Amersham International PLC (Little Chalfont, UK). IFN-α and IFN-γ were obtained from R & D Systems (Minneapolis, MN). All measurements were performed using a Beckman DU 640 spectrophotometer, a Beckman-LS5000TD liquid scintillator (Beckman Instruments, Fullerton, CA), and a Shimadzu high-performance liquid chromatography (HPLC) system (Tokyo, Japan).

[1] Correspondence: Departamento de Histologia e Embriologia, Instituto de Ciências Biomédicas I, Universidade de São Paulo, Av. Lineu Prestes, 1524, sala 302, 05508-900, Butantan, São Paulo, Brasil. E-mail: ggrosa@icb.usp.br

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Cell obtainment

Phosphate-buffered saline, pH 7.2, was injected (6.0 mL) intraperitoneally, and after 30 s, peritoneal macrophages were collected with a Pasteur pipette. The samples were centrifuged against a Ficoll-hypaque gradient (Hystopaque® 1077), and the mononuclear cells were separated. Cell viability was confirmed by trypan blue exclusion (>95%).

Serum from tumor-bearing (TB serum) animals

A Walker-256 cell suspension (approximately 1×10^7 in 1.0 ml) was injected subcutaneously into the left flank of the rats. After 15 days, the rats were killed, and the serum was obtained after whole blood centrifugation.

Incubation procedure

Macrophages obtained from eight rats were pooled together to reach the final concentration of 1.0×10^7/flask. Cells from the control group were incubated at 37°C in Krebs Ringer medium with 2% fat-free bovine serum albumin in the presence of radiolabeled tryptophan (50 mM, 5 μC), glucose (5 mM), and glutamine (2 mM). Some cells were cultivated in the presence of IFN-α, 90 U/mL; IFN-γ, 10 U/mL; LPS, 20 ng/mL; PMA, 0.8 nM; or 100 μL Walker-256 TB rats serum. After 1.5 h, the supernatant was collected and stored at –70°C for the measurement of tryptophan, melatonin, and serotonin. Cells were disrupted with 200 μL trichloracetic acid (25% w/v), and the samples were neutralized with 100 μL KOH and tris-(hydroxymethyl)aminomethane/KOH (0.5 M/2.0 M) solution and frozen for the measurements of the maximal activity of the arylalkylamine N-acetyltransferase (AA-NAT).

Determination of tryptophan, melatonin, and serotonin

The content of melatonin, serotonin, and tryptophan in cell incubation supernatant was determined by HPLC with electrochemical detection (method modified from Cipolla-Neto et al., ref. [19]). The chromatographic system (Shimadzu, Kyoto, Japan) was composed by an isocratic LC-10AD vp HPLC pump, a resolve 5 μm spherical C18, 3.9 × 150 mm steel column, and a L-ECD-6A electrochemical detector operated in DC mode, controlled by the Shimadzu CLASS-VP software through a system interface module.

Each sample was centrifuged (14,000 *g*, 2 min, Eppendorf 5415C centrifuge, Brinkmann Instruments, Westbury, NY), and the clear supernatant (60 μL) was injected into the system through a syringe-loading injector (20 μL loop, Model 7125, Rheodyne, Rohnert Park, CA).

The chromatographic system was operated with the following phase at 30°C: 0.1 M sodium acetate, 0.1 M citric acid, 0.15 M EDTA, 35% methanol, pH 3.7, at a constant flow rate of 1.0 mL/min. The detector potential was adjusted to a steady value of +900 mV (vs. Ag/AgCl reference electrode). The total run time was 30 min. The peaks were quantified by measuring the peak area on the chromatogram. Calibration curves were made under the same conditions described using different amounts of each standard sample in aqueous solution. The detection limit was defined as the lowest injected amount that produced a signal-to-noise ratio of three (tryptophan, 35 ng/mL; serotonin, 9.7 ng/mL; melatonin, 0.5 ng/mL).

Radioimmunoassay for melatonin

Melatonin concentration of supernatants was also measured in duplicate by radioimmunoassay using [^{125}I] melatonin (Amersham, Bucks, UK; specific activity, 2000 Ci/mmol). The standard range of dilutions extended from 3.75 pg/mL to 4 ng/mL, and the minimum detection limit level was 4 pg/mL. The intra-assay coefficients of variation were 18%, 10%, and 10% for standards containing 50, 250, and 1250 pg/mL, respectively, and the interassay coefficient of variation among seven assays was 18%, 10%, and 5% for the three concentrations mentioned before [20].

Radiolabeled tryptophan uptake

Samples of 10 μL supernatant of the incubated cells were collected at 1, 5, 10, 50, and 90 min of incubation and were added to 1.0 mL Ecolume liquid scintillation cocktail. Radioactivity was measured in a Beckman-LS5000TD liquid scintillator. Previous measurements show that under our conditions, less than 3% of the total amount of labeled tryptophan binds to cell membrane. We also noticed that when radiolabeled tryptophan was incubated in the presence of cell homogenate previously deproteinized, there was no significant degradation.

Enzyme activity

AA-NAT (EC 2.3.1.5) maximal activity was measured in peritoneal macrophages following the method described by Parfitt and colleagues [21] through a radioenzymatic assay. Cells, after disruption, were incubated in a shaking water bath at 37 ± 0.5°C for 20 min in a medium containing phosphate buffer 0.1 M, pH 6.8, tryptamine 40 mM, and [^{3}H]acetyl-CoA 2 mM. The reaction was stopped by the addition of 250 μL ice-borate buffer (pH 10). Chilled chloroform (1 mL) was added. The tube was swirled for 1 min at room temperature and centrifuged at 3000 *g*, 20 s. The aqueous phase was removed, 500 μL chloroform and 1 mL scintillation cocktail (Insta-Fluor-Packard®) were added, and radioactivity was counted in a Beckman® LS6500 β counter.

Statistical analysis

The results (ng melatonin/mg protein) are expressed as mean ± SD. All data were analyzed using the GraphPad Prism program and graph package (V3.0, GraphPad, San Diego, CA). Groups were compared by ANOVA and the Turkey post-test.

RESULTS

Macrophages obtained from the peritoneal cavity of Wistar rats, when incubated for 1 h in the presence of tryptophan (500 nM), produced 5-HT (141.5±8.7 nmol/h per mg protein) and melatonin (260.5±16.2 nmol/h per mg protein; **Table 1**). The incubation of the cells in the presence of LPS, PMA, and serum from Walker-256 serum TB rats increased melatonin production by 31%, 48%, and 52%, respectively, and decreased that of 5-HT by 26% and 35% for LPS and PMA (Table 1). The presence of TB rat plasma did not change 5-HT production by macrophages (Table 1). The presence of IFN-γ or IFN-α reduced melatonin production by 48% and 25%, respectively, enhancing that of 5-HT by 108% and 41% for IFN-γ or IFN-α, respectively (Table 1).

The production of melatonin by incubated macrophages started 20 min after the beginning of tryptophan consumption, as shown in **Figure 1**, and was linear from that point until the end of the 1-h period (tryptophan consumption, $R^2=0.94$; melatonin production, $R^2=0.86$ for the 1-h period and $R^2=0.99$ for the last 40 min; Fig. 1). The addition of IFN-γ to the culture medium increased tryptophan consumption by 31%

TABLE 1. Melatonin and Serotonin Production

	5-HT		Mel	
No addition	141.5	8.7	260.5	16.2
IFN-γ	308.5*	30.4	132.7*	9.6
IFN-α	199.5*	4.5	197.5*	25.6
LPS	103.6*	9.4	342.3*	24.1
PMA	98.5*	8.7	385.8*	22.9
serum TB	137.8	9.5	397.5*	31.7

Melatonin (Mel) and serotonin (5-HT) production (ng/mL per mg protein) by peritoneal cavity macrophages incubated for 1 h in the presence of tryptophan 50 mM and IFN-α 90 U/mL or -γ 10 U/mL, LPS 20 ng/mL, PMA 0.8 nM, or 100 μL Walker-256 serum TB. The results represent mean ± SEM of three experiments in triplicates. * $P < 0.05$ for comparison with values obtained for the control group.

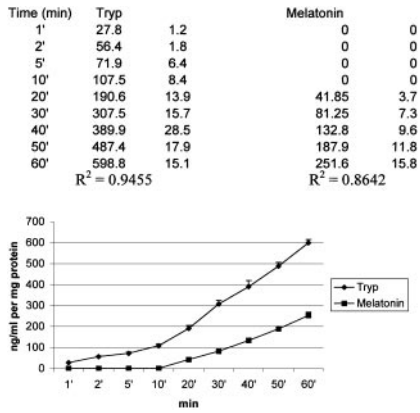

Fig. 1. Tryptophan consumption (Tryp) and melatonin production (ng/mL per mg protein) by peritoneal cavity macrophages incubated for 1 h in the presence of tryptophan 50 mM. The results represent mean ± SEM of five experiments in triplicates.

(R^2=0.92; **Fig. 2**) and reduced melatonin production by 47% (R^2=0.90; Fig. 2) when compared with the results shown in Figure 1.

The addition of tryptophan (5 μM) increased AA-NAT maximal activity by 11.2-fold for cells obtained from control rats (**Table 2**). It is interesting that PMA, when added to the incubation medium, did not enhance AA-NAT activity in the cells from control rats (Table 2); IFN-γ reduced the enzyme activity by 32% (Table 2), and LPS and the serum from TB rats increased it by 28% and 21%, respectively (Table 2).

Melatonin production by peritoneal macrophages was influenced by the amount of tryptophan added to the medium, as shown in **Figure 3**. In fact, when tryptophan was added at physiological plasma concentration (50 μM, ref. [22]), melatonin production reached the peak after 20 min. The presence of tryptophan 500 μM induced a more pronounced melatonin production, which reached the peak after 50 min incubation, and tryptophan 50 mM led to a maximal production of melatonin after 60 min incubation (Fig. 3).

DISCUSSION

Neuroendocrine regulatory factors play a fundamental role in the development and function of the immune system and regulate adaptive and natural immunity [23]; many substances produced by immune cells act as feedback signals toward the neuroendocrine system [24, 25], and some of them are also produced in many organs and tissues, including the central nervous system [26]. It is also apparent that leukocytes are capable of producing classic hormones and neuropeptides [27]. Among the hormones involved in neuroimmunomodulation, there is a large scope of evidence that points out melatonin as a strong candidate, as it presents marked effects on the neuroendocrine system, is produced by leukocytes [5–8], and plays a complex, physiological role in maintaining the homeostasis of the immune system [28–30]. The production of melatonin by PBML opens the possibility to the existence of a two-way pathway within the neuroendocrine system [7].

Our data pointed out that tryptophan consumption by macrophages increased as the amino acid availability augmented from 50 μM, a physiological concentration of the amino acid in the plasma [20] when it reached the peak at 30 min of incubation, until 50 mM, when the consumption was linear (R^2=0.94) over 1 h of incubation. As we found a linear consumption of tryptophan at 50 mM, we chose this concentration to be used in the other experiments. From the total amount of tryptophan offered to the cells over 1 h, the consumption of the amino acid represented 10% of the total amount present in the incubation medium, opening the possibility to the action of other tryptophan metabolism regulatory mechanisms when necessary.

The consumption of tryptophan by monocyte-derived macrophages and the release of kynurenine metabolites were extensively reported in the literature as an important immunoregulatory mechanism [31–34], as the reduction of tryptophan availability decreases T and NK cell proliferation [33, 35] as well as the production of inflammatory and immunological mediators [31, 33, 36]. However, other tryptophan metabolites such as serotonin and related compounds might also influence immune and inflammatory responses in ways not yet defined [35].

Macrophages obtained from the peritoneal cavity of normal rats diverted 42% of the tryptophan consumed in 1 h of incubation to melatonin synthesis, reinforcing the ability of such cells in producing the indolamine, as previously published by Finocchiaro and colleagues [7]. In such cells, melatonin could be oxidized through myeloperoxidase-producing kynurenines, as described by Rodrigues and colleagues [37], opening another pathway for understanding the immunoregulatory function of tryptophan.

Considering that IFN-α and -γ, LPS, and PMA stimulate tryptophan metabolism in leukocytes, leading to increased production of kynurenines and/or serotonin and related metabolites [32, 35, 38, 39], we evaluated the effect of such compounds on melatonin and serotonin production by macrophages incubated with tryptophan. In fact, we observed that the treat-

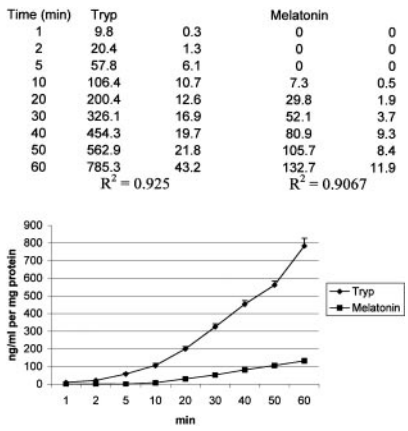

Fig. 2. Tryptophan (Tryp) consumption and melatonin production (ng/mL per mg protein) by peritoneal cavity macrophages incubated for 1 h in the presence of tryptophan 50 mM and IFN-γ (10 U/mL). The results represent mean ± SEM of five experiments in triplicates.

TABLE 2. AA-NAT Maximal Activity in Macrophages

NAT	Control	Tryp	Tryp + PMA	Tryp + LPS	Tryp + IFN-γ	Tryp + serum TB
	4.03 ± 0.84	45.3 ± 5.31*	44 ± 13*	57.9 ± 6.4*,[a]	30.8 ± 4.1*,[a]	54.8 ± 7.4*,[a]

AA-NAT (NAT) maximal activity in macrophages obtained from the peritoneal cavity of normal rats incubated for 1 h in the presence of tryptophan (TryR; 50 mM) or tryptophan plus PMA (0.8 nM). The results are expressed as mM/min per mg protein and represent the mean ± SEM of five experiments. * $P < 0.05$ for comparison with values obtained for the control group. [a] $P < 0.05$ for comparison with the values obtained for the Tryp group.

ments did not interfere with the total amino acid uptake by macrophages (approximately 9%) but substantially changed the profile of serotonin and melatonin production. IFN-γ reduces melatonin production, diverting tryptophan metabolism toward serotonin, which concentration increases 108%. This increase in 5-HT concentration could be related to a decrease in the maximal activity of AA-NAT and hydroxy-indole-O-methyltransferase (HIOMT), as these are the regulatory steps in melatonin synthesis. The addition of IFN-α slightly increased serotonin concentration and reduced that of melatonin, indicating also a decreased flux of substrates through the enzymes AA-NAT and HIOMT. The other treatments, PMA and LPS, reduced serotonin concentration, increasing the amount of 5-HT diverted to melatonin synthesis. These results strongly suggest the existence of a regulatory mechanism involving the modulation of the serotonin/melatonin ratio at the inflammatory site.

It is interesting that LPS, which has been shown to synergize with melatonin to activate macrophages [40], induces an increase in melatonin production and reduction of serotonin synthesis. Conversely, IFN-γ increased serotonin concentration and reduced melatonin synthesis as part of an immunoregulatory circuit proposed by Arzt and colleagues [14], through which the increase in serotonin production stimulated by IFN-γ controls IFN production by such cells in a negative-feedback pattern. Therefore, our data strongly suggest the existence of other immunoregulatory mechanisms concerning serotonin–melatonin and tryptophan and cytokine production with stimulatory and inhibitory loops, as shown in **Figure 4**. The existence of these regulatory mechanisms does not exclude that which involves the production of kynurenines through indoleamine 2,3 dioxygenase and is related to immunosupression in many different circumstances as during pregnancy [35], as the total amount of tryptophan diverted to melatonin and serotonin synthesis in our study was approximately 10% of the total amount of the amino acid.

Cancer cachexia syndrome is clinically characterized by anorexia, wasting, weight loss, weakness, fatigue, poor performance status, and impaired immune function [41]. Many clinical and biological studies have demonstrated that the syndrome may depend on alterations of neuroendocrine and cytokine secretions. Considering that melatonin is a potent candidate to be mediating neuroimmunoendocrine relations during cachexia, by its control on the bidirectional communication between the neuroendocrine and immune system [1, 5, 6], we have also evaluated the effect of TB rat serum on the production of melatonin and serotonin by peritoneal macrophages. The same pattern of response found in the presence of PMA or LPS was observed when macrophages were incubated with the serum obtained from TB rats. This increase in mela-

Time (min)	Tryp 50 μM		Tryp 500 μM		Tryp 50 mM	
1'	0.5	0.1	22.1	1.4	27.8	1.2
2'	9.4	0.5	41.3	2.5	56.4	1.8
5'	16.4	0.8	62.7	2.9	71.9	6.4
10'	27.6	1.7	100.5	9.4	107.5	8.4
20'	48.7	2.9	185.9	15.6	190.6	13.9
30'	50	0	299.4	14.2	307.5	15.7
40'	50	0	381.8	17.8	389.9	28.5
50'	50	0	472.1	16.4	487.4	17.9
60'	50	0	488.9	21.7	598.8	15.1
	$R^2 = 0.8422$		$R^2 = 0.9542$		$R^2 = 0.9455$	

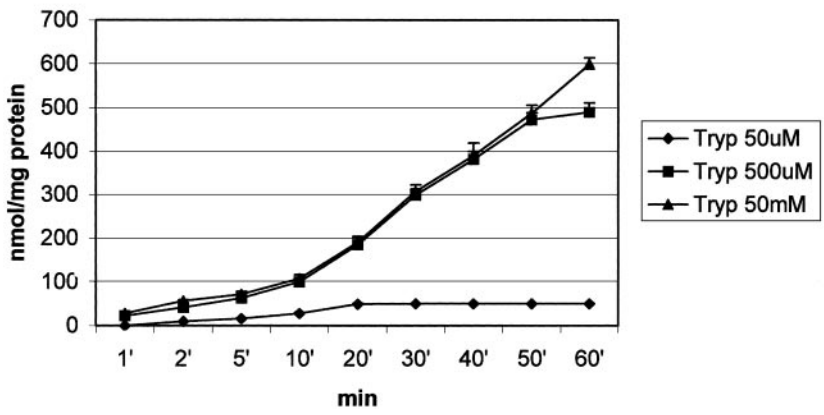

Fig. 3. Consumption of tryptophan (Tryp) by peritoneal cavity macrophages incubated for 1 h in different concentrations of tryptophan (50 μM, 500 μM, and 5 mM). Results are expressed in nmol/mg protein and represent the mean ± SEM of five experiments in duplicates.

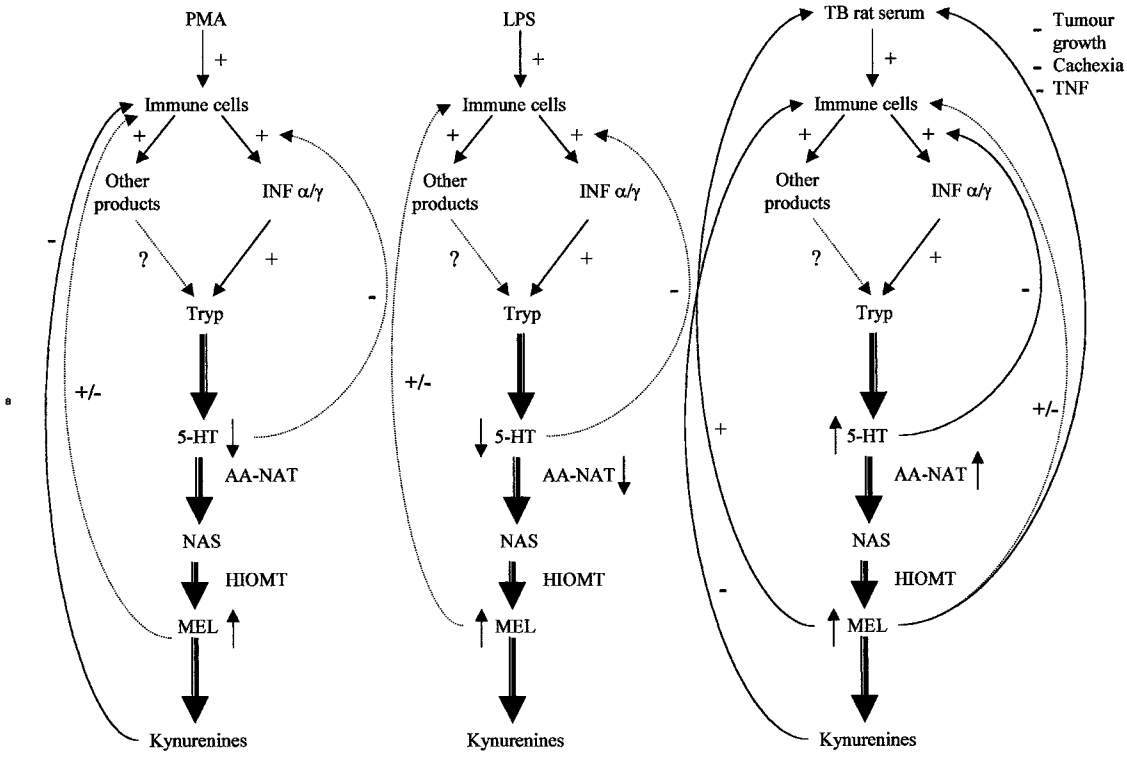

Fig. 4. Schematic view of the possible regulatory pathways proposed for macrophages incubated in the presence of LPS, PMA, or serum from TB rats. Tryp, Tryptophan; NAS, N-acetylserotonin; MEL, melatonin; TNF, tumor necrosis factor; INF α/γ, IFN-α/-γ.

tonin synthesis could be part of another immunoregulatory axis, as melatonin inhibits TNF production in advanced cancer patients [8], a response that could attenuate cachexia or inhibit tumor growth [42–44]. Conversely, tumor growth seems to modulate pineal secretory activity [45].

Considering that the ratio of serotonin/melatonin seems to be part of an immunoregulatory mechanism, we evaluated the maximal activity of AA-NAT, a key step in melatonin synthesis. It is interesting to note that tryptophan, when added to the incubation medium, increased AA-NAT maximal activity and that the presence of PMA, which increased melatonin production by 48%, did not alter the activity of the enzyme. Conversely, LPS and the serum of TB rats increased the maximal activity of the enzyme, diverting more tryptophan to melatonin synthesis, and IFN-γ, when added to the incubation medium, reduced it by 32%. It seems that in the presence of tryptophan, the enzyme reaches its maximal activity and that in such condition, other sites such as HIOMT could be acting as controllers of melatonin synthesis. These possible changes in serotonin/melatonin synthesis pathways deserve to be better evaluated.

Our results indicate that macrophages obtained from the peritoneal cavity of normal rats when incubated with tryptophan show an increase in AA-NAT activity that corresponds to an increased melatonin production, as determined in the incubation medium. This process is regulated by IFN-α and -γ, PMA, LPS, and the serum from TB rats, opening the possibility of speculation about different immunoregulatory loops acting through the balance of melatonin/serotonin production by such cells.

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