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# The effect of melatonin chronic treatment upon macrophage and lymphocyte metabolism and function in Walker-256 tumour-bearing rats

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**Abstract**

Melatonin is the main hormone involved in the neuroendocrine-immune axis. It also presents antitumour activity. To evaluate the role of melatonin on the progression of Walker-256 tumour in rats we determined the effect of the hormone on some biochemical and functional aspects of macrophage and lymphocytes from cachectic rats. An important finding observed in immune cells from tumour-bearing (TB) rats is the impairment on glutamine and glucose metabolism in such cells. These changes are very similar to those observed in pinealectomized rats (PNX). The increased production of lactate and the flux of glucose through the Krebs cycle and the reduction in glutamine consumption seems to be involved in the immunosuppression presented in the TB and PNX animals. Melatonin treatment restored the changes observed in the metabolism of glucose and glutamine and stimulated the proliferation of lymphocytes from tumour-bearing rats. The results indicate that the effect of melatonin upon tumour growth involves the stimulation of the immune system by the hormone. © 1998 Elsevier Science B.V.

*Keywords:* Melatonin; Immune system; Tumour; Glucose and glutamine

## 1. Introduction

Recently, several experiments have been performed to identify the mechanism(s) responsible for bi-directional communication between the neuroendocrine and immune systems (Weigent and Blalock, 1995). The link between these systems depends on several immune factors, hormones and common receptors (Blalock, 1992, 1994a,b). Among these, a possible candidate, in whose favour a large range of evidence has been described (Calvo et al., 1995; Maestroni, 1995; Finocchiaro et al., 1991; Lissoni et al., 1993), is the neurohormone melatonin.

Melatonin has been shown to regulate several immune functions, such as natural killer cell cytotoxicity (Hellstrand and Hermodsson, 1987, 1990), antibody production (Jackson et al., 1985; Maestroni et al., 1987a,b), lymphocyte proliferation, IFN-$\gamma$ production (Artz et al., 1988) and T-helper-2 function (Calvo et al., 1995). Some experiments have also demonstrated an indirect effect of melatonin upon the immune system through the endogenous opioid system, which is able to control macrophage and lymphocyte function (Maestroni and Conti, 1991; Panerai et al., 1991) or through an effect upon bone marrow. On the other hand, Finocchiaro et al. (1991) showed that peripheral blood mononuclear leukocytes have the capacity to metabolize 5-HT to melatonin, opening up the possibility of the existence of a two-way path in the neuroendocrine system.

Among the functions delegated to melatonin in the control of the immune system we could include host antitumour defenses (Regelson and Pierpaoli, 1987; Maestroni et al., 1988a,b, 1990). The hormone, however, seems to act only in immunologically primed cells, acting synergistically with IL-2 in lymphocytes (Lissoni et al., 1993). Pinealectomy stimulates the growth of a few tumours (Bennet, 1985) and the injection of melatonin provoked a reduction in that rate of growth (Cardinali et al., 1979). Besides the effect upon lymphocytes, melatonin is shown to activate monocytes/macrophages, inducing in this cell

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type, the production of interleukin-1 and reactive oxygen intermediates and the response of macrophages to lipopolysaccharide stimulation (Poon and Pang, 1992). The combined effect of melatonin upon lymphocyte and macrophage function could be related to the antitumour properties of the neurohormone. Little is known, however, about the effect of melatonin upon lymphocyte and macrophage metabolism, or about its effect upon the progression of Walker-256 carcinosarcoma.

Lymphocytes are nucleated rapidly dividing cells, which utilize high rates of glucose and glutamine (Curi et al., 1986; Newsholme et al., 1989). The partial metabolization of these substrates allows the cells to present high rates of fatty acid and protein synthesis, as well as high rates of energy production. Macrophages, however, are fully differentiated cells, that present a high secretory activity, which includes products of proteic and lipidic structure. To cope with their secretory activity, macrophages, similarly to lymphocytes, show the same metabolic pattern, utilizing high rates of glucose (aerobic glycolysis) and glutamine (glutaminolysis) (Newsholme et al., 1987). In the present study we have evaluated the effect of melatonin upon glucose and glutamine metabolism in macrophages and lymphocytes from Walker-256 tumour-bearing rats and the effect of the neurohormone or its absence (pinealectomized rats) upon tumour progression as a measurement of its indirect influence upon the macrophages and lymphocytes.

The metabolic changes induced by melatonin in lymphocytes and macrophages were investigated by measuring different parameters of metabolic pathways in these cell types. Among those analyzed are the maximal activity of hexokinase, citrate synthase, glucose-6-phosphate dehydrogenase and glutaminase, as well as the rate of $^{14}CO_2$ produced by the oxidation of [U-$^{14}$C]glucose and [U-$^{14}$C]glutamine, through the Krebs cycle.

## 2. Material and methods

### 2.1. Animals

Adult male Wistar rats, weighing approximately 150–200 g, were obtained from the Animal Breeding Unit, Institute of Biomedical Sciences, University of Sao Paulo, where they were housed in a temperature controlled room at 23°C under a 12 h light/12 h dark cycle, lights on at 8:00 p.m.

### 2.2. Reagents

The buffer reagents were obtained from Reagen Quimibras Industria Brasileira SA, and the others were obtained from Boehringer Mannheim, Lewes, East Sussex, and Sigma, St. Louis, MO, except for [U-$^{14}$C]glucose and [U-$^{14}$C]glutamine which were from Amersham, UK. All measurements were performed using a Gilford (Model Response) and a Hitachi U2001 spectrophotometer.

### 2.3. Experimental design

To address the effect of melatonin upon the metabolism and function of macrophages and lymphocytes from tumour-bearing rats we studied the following groups:

Control-rats without any kind of treatment;

PNX-normal rats submitted to pinealectomy;

PNX-MEL-pinealectomized rats treated with a daily dose of melatonin;

TB rats inoculated subcutaneously with a suspension of Walker-256 tumour cells on the left flank;

TB-MEL rats inoculated subcutaneously with a suspension of Walker-256 tumour cells on the left flank that received, daily, a dose of melatonin;

TB-PNX-pinealectomized rats that had been submitted to the tumour implantation, to evaluate the effect of the absence of the diurnal peak of endogenous melatonin upon the immune system in TB rats;

TB-PNX-MEL-pinealectomized rats that had been submitted to the tumour implantation, that received, daily, a dose of melatonin.

The rats received 0.3 ml of melatonin (1 mg/ml) every day, 1 h before the beginning of the dark period. The tumour-bearing rats were treated during the 14 days of tumour development. The other groups were treated during the same period of time.

### 2.4. Pinealectomy

The animals were anesthetized with pentobarbital (45 mg/kg b.w.) and submitted to the surgery, according to Hoffman and Reiter (1965). In brief, the anesthetized animals were placed in a stereotaxic apparatus for small animals and a sagittal opening was on the scalp. The skin and muscles were pushed aside in order to expose the lambda suture. By means of a circular drill a disc-shaped perforation was done around the lambda and the disc-shaped piece of bone was delicately removed. Thereafter, the pineal gland (which is located just below the posterior venous sinus confluence) was pulled out with fine forceps. After a brief period of haemostasis, the skull was closed by returning the disc-shaped bone and the scalp was sutured with cotton threads.

### 2.5. Tumour implantation

A Walker-256 cell suspension (approx. $1 \times 10^7$ in 1.0 ml) was injected s.c. into the left flank of the rats, and the controls were injected with 1.0 ml 0.9% (w/v) NaCl without anaesthesia. The amount injected ensured that the tumour mass was 8–10% of the carcass weight at the time of the experiments. All experiments were commenced between 08:00 and 10:00 a.m., 14–15 days following the tumour implantation.

### 2.6. Peritoneal cell preparation

Cells present in the intraperitoneal cavity of the rats were collected using 6 ml phosphate-buffered saline (PBS) and considered as resident macrophages (unstimulated). Cell viability was confirmed by Trypan blue exclusion (> 95%). In all experiments, at least 92% of the peritoneal exudate cells were macrophages as determined by differential counting.

### 2.7. Lymphocyte preparation

The cells were obtained as previously described (Curi et al., 1989; Vieira et al., 1990) from the mesenteric lymph nodes and spleen pressed against a steel screen. Peripheral blood lymphocytes were obtained by the centrifugation of the blood against a gradient of Percoll, density 1.077. The cell suspension was then filtered (Whatman 105 cat No. 2105841 filter, UK) and centrifuged at 1500 rpm for 15 min at 4°C. The pellet was resuspended in the extraction buffer, which was specific for each enzyme, as described in Section 2.12, or plated for culture procedures. The population of cells obtained consisted of T and B lymphocytes and the total contamination with macrophages was lower than 1% (Almeida et al., 1989).

### 2.8. Incubation procedure

Macrophages and lymphocytes were incubated ($1.0 \times 10^6$ cells/flask) at 37°C in Krebs–Ringer medium with 2% fat-free bovine serum albumin and in the presence of glucose (5 mM) or glutamine (2 mM). After incubation, the cells were disrupted by the addition of 0.2 ml of 25% perchloric acid. Protein was removed by centrifugation and the supernatant was neutralized with 20 $\mu$l of a 40% KOH solution and a Tris-(hydroxymethyl) aminomethane/KOH (0.5–2.0 M) solution for the measurement of the metabolites (Costa Rosa et al., 1993).

### 2.9. Metabolite measurements

Neutralized samples of the incubation medium were analyzed for the measurement of lactate (Engle and Jones, 1978), glucose (Bergmeyer et al., 1974) and glutamine (Windmueller and Spaeth, 1974). The method for glucose determination is based on the conversion of glucose to glucose-6-phosphate by hexokinase and the subsequent formation of 6-phosphogluconate from glucose-6-phosphate by glucose-6-phosphate dehydrogenase with production of NADPH, as reported by Bergmeyer et al. (1974). The lactate in the neutralized samples was measured using the method of Engle and Jones (1978), through the reaction of lactate dehydrogenase, in which the conversion of lactate to pyruvate produces NADH. The production of NADH and NADPH was monitored at 340 nm, in a spectrophotometer (Hitachi model U2001). Glutamine consumption was measured by following the conversion of $\alpha$-ketoglutarate into glutamate that utilizes one $NH_3$ molecule from the conversion of glutamine into glutamate in the presence of asparaginase. The conversion of $\alpha$-ketoglutarate into glutamate that is catalyzed by the enzyme glutamate dehydrogenase is accompanied by the conversion of one NADH to one NAD. By comparing the values at time zero and those obtained after 1 h incubation it was possible to calculate glucose and glutamine consumption and lactate production by the incubated macrophages and lymphocytes.

The $^{14}CO_2$ produced from [U-$^{14}$C]glucose and [U-$^{14}$C]glutamine was collected as previously described (Kowalchuk et al., 1988). Macrophages and lymphocytes were incubated for 1 h in the presence of one of the radiolabelled substrates in a sheltered Erlenmeyer (25 ml), containing one compartment for cell incubation and another for $CO_2$ collection. After this period of time the cells were killed using 200 $\mu$l of a perchloric acid solution (25%). The labelled $CO_2$ was collected for 1 h in a solution of phenylethylamine:methanol (1:1) and the radioactivity counted, using Bray's scintillation cocktail, consisting of 60 g/l naphthalene, 4 g/l 2,5-diphenyloxazole (PPO), 200 mg/l 1,4-di-[2-(5-phenyloxazolyl)]-benzene (POPOP), 10% methanol (by vol.) and 2% ethylene glycol (by vol.) in *p*-dioxan (chromatographic grade), in a Beckman-LS 5000TD liquid scintillator (Beckman Instruments, Fullerton, CA). All reagents used in the preparation of Brays were obtained from Sigma (USA) or Merck (Darmstadt).

### 2.10. Macrophage phagocytosis

Macrophages were incubated with 10 ml PBS containing opsonized zymosan for 30 min at 37°C so that phagocytosis could be quantified by counting (in a counting chamber) the percentage of cells that had phagocytosed more than four particles of zymosan (Costa Rosa et al., 1996b).

### 2.11. Hydrogen peroxide production

The production of $H_2O_2$ was measured by a modification of the method described by Pick and Mizel (1981). The cells were incubated in siliconized flasks (25 ml), in 1 ml PBS, in the presence of glucose (5 mM), under an atmosphere of 5% $CO_2$/95% air at 37°C and in the presence of phorbol-myristate-acetate (PMA) (10 ng/ml) when indicated. After 1 h incubation, a solution of phenol red and horseradish-peroxidase (HRPO) was added to the medium to quantify the hydrogen peroxide content. After 10 min the reaction was stopped with 100 $\mu$l of 1 N NaOH and the amount of hydrogen peroxide formed was measured spectrophotometrically at 620 nm.

### 2.12. Enzyme assays

Enzyme activities were measured as previously described (Cooney et al., 1981; Cooney and Newsholme, 1982; Stanley and Newsholme, 1985; Curi et al., 1988). The extraction medium for hexokinase (EC 2.7.1.1) contained 25 mmol Tris–HCl/l, 1 mmol EDTA/l and 30 mmol $\beta$-mercaptoethanol/l at pH 7.4 and that for glutaminase (EC 3.5.1.2) contained 150 mmol potassium phosphate/l, 1 mmol EDTA/l and 50 mmol Tris–HCl/l at pH 8.6. The extraction medium for citrate synthase (EC 4.1.3.7) contained 50 mmol Tris–HCl/l and 1 mmol EDTA/l; the final pH was 7.4. To all enzyme assays, 0.05% (v/v) Triton X-100 was added to complete the extraction of the enzymes. For the assay of hexokinase (the activity of the enzyme was measured through the production of NADPH via glucose-6-phosphate dehydrogenase), the following medium was used: 75 mmol Tris–HCl/l, 7.5 mmol $MgCl_2$/l, 0.8 mmol EDTA/l, 1.5 mmol KCl/l, 4.0 mmol $\beta$-mercaptoethanol/l, 0.4 mmol creatine phosphate/l, 1.8 U creatine kinase, 1.4 U glucose-6-phosphate dehydrogenase, 0.4 mmol NADP/l, pH 7.5. The assay of citrate synthase (the enzyme activity was determined following the production of a yellow compound which results from the reaction between DTNB and the CoA released) consisted of 100 mmol Tris–HCl/l, 0.2 mmol 5,5′-dithiobis-2-nitrobenzoic acid, 15 mmol acetyl-CoA/l and 0.5 mmol oxaloacetate/l, pH 8.1. The assay medium for glutaminase (the activity of the enzyme was evaluated through the production of NADH in the conversion of glutamate to $\alpha$-ketoglutarate catalyzed by glutamate dehydrogenase) consisted of 50 mmol phosphate buffer/l (equimolar mixture of $KH_2PO_4$ and $K_2HPO_4$), 0.2 mmol EDTA/l, 50 mmol Tris–HCl/l, 20 mmol glutamine/l, pH 8.6. The final volume of the assay mixtures in all cases was 1.0 ml. Citrate synthase was assayed by following the rate of change in absorbance at 412 nm, and the remainder of the enzymes was assayed by following the rate of change in absorbance at 340 nm. All spectrophotometric measurements were performed at 25°C, except for glutaminase which was determined at 37°C. Preliminary experiments established that extraction and assay procedures were such as to produce maximum enzyme activities (Crabtree et al., 1979) for all enzymes studied.

### 2.13. Incorporation of [2-$^{14}$C]thymidine into cultured lymphocytes

Lymphocytes were cultured in RPMI-1640 medium for 48 h at 37°C in an artificially humidified atmosphere of 5% $CO_2$ in air under sterile conditions. Cultures were performed in a LAB-LINE Microprocessor $CO_2$ incubator (Lab Line, USA) in 96-well plates (Corning, NY, USA), $1 \times 10^5$ cells per well (total volume, 200 $\mu$l). Cells were also cultivated in the presence of concanavalin-A (5 $\mu$g/ml), a mitogen for T cells, or lipopolysaccharide (10 mg/ml), a mitogen for B cells. After 48 h in culture, more than 98% of lymphocytes were viable, as measured by trypan blue dye exclusion. The cells were then pulsed with 20 $\mu$l of 0.02 $\mu$Ci [2-$^{14}$C]thymidine (sp. act. 56.0 mCi/nmol), diluted in sterile PBS yielding a final concentration of 1 $\mu$g/ml. Cells were then maintained under these conditions for an additional 15 h and automatically harvested by using a multiple cell harvester and filter papers cat. No. 11731 (Skatron Combi, Sufolk). The paper discs containing labelled cells were counted in 5 ml of Bray's scintillation cocktail, in a Beckman-LS 5000TD liquid scintillator (Beckman Instruments, Fullerton, CA).

### 2.14. Expression of the results

The enzyme activities are expressed as nmol substrate utilized/min/mg protein. The rates of glucose and glutamine consumption, lactate formation and of decarboxylation of [U-$^{14}$C]glucose and [U-$^{14}$C]glutamine are presented as nmol/h/mg protein.

### 2.15. Statistical analysis

Results are expressed as mean ± S.E.M. The paired *t*-test and ANOVA were employed and differences were considered significant at $P < 0.05$.

## 3. Results

Pinealectomy per se, induced a decrease in hexokinase (HK) activity (30%), as well as citrate synthase (CS −48%) and glucose-6-phosphate dehydrogenase (G6PDh −35%,

Table 1
Maximal activities of hexokinase (HK), citrate synthase (CS), glucose-6-phosphate dehydrogenase (G6PDh) and glutaminase (GLUT) in lymphocytes obtained from tumour-bearing (TB) and pinealectomized tumour-bearing rats (PNX-TB) and control rats (C) treated with a daily dose of melatonin (MEL)

| Ly | C | PNX | PNX-MEL | TB | TB-MEL | PNX-TB | PNX-TB-MEL |
|---|---|---|---|---|---|---|---|
| HK | 62.4 ± 2.1 | 43.1 ± 2.1* | 64.2 ± 1.3 | 25.1 ± 1.1* | 60.1 ± 1.7 | 35.3 ± 3.1* | 64.0 ± 2.4 |
| CS | 294.3 ± 17.2 | 153.2 ± 9.8* | 281.0 ± 7.0 | 174.8 ± 11.3* | 268.1 ± 17.4 | 153.4 ± 8.9* | 284.2 ± 17.8 |
| G6PDh | 81.3 ± 6.4 | 52.3 ± 3.9* | 86.4 ± 4.1 | 50.2 ± 4.8* | 48.3 ± 3.4* | 88.4 ± 3.7 | 74.3 ± 1.3* |
| GLUT | 68.4 ± 1.0 | 69.4 ± 1.1 | 62.9 ± 3.5 | 67.2 ± 7.0 | 60.4 ± 3.1 | 94.3 ± 4.2* | 72.4 ± 4.3 |

The results are expressed as nmol/min/mg protein and represent the mean ± S.E.M. of 7 experiments.
* $P < 0.05$ for comparison with the control group.

Table 2
Glucose and glutamine consumption (C-GLUC and C-GLUT, respectively) and their decarboxylation rate (D-GLUC and D-GLUT) and lactate production in lymphocytes obtained from tumour-bearing (TB) and pinealectomized tumour-bearing rats (PNX-TB) and control rats (C) treated with a daily dose of melatonin (MEL)

| Ly | C | PNX | PNX-MEL | TB | TB-MEL | PNX-TB | PNX-TB-MEL |
|---|---|---|---|---|---|---|---|
| C-GLUC | 68.2 ± 4.1 | 52.1 ± 2.7 * | 71.8 ± 2.9 | 48.3 ± 3.0 * | 73.2 ± 2.7 | 34.3 ± 3.1 * | 61.0 ± 2.8 |
| LACT | 103.6 ± 3.0 | 93.5 ± 1.2 | 101.2 ± 5.2 | 38.3 ± 5.3 * | 98.3 ± 7.5 | 34.6 ± 4.4 * | 85.4 ± 8.7 |
| D-GLUC | 12.6 ± 0.7 | 4.5 ± 0.4 * | 11.7 ± 1.7 | 24.1 ± 1.1 * | 12.3 ± 2.1 | 15.3 ± 2.6 | 11.3 ± 1.7 |
| C-GLUT | 70.4 ± 2.7 | 54.2 ± 2.1 * | 75.3 ± 1.9 | 94.3 ± 5.7 * | 68.3 ± 2.4 | 121.4 ± 8.7 * | 76.3 ± 1.4 |
| D-GLUT | 36.8 ± 0.9 | 26.8 ± 1.1 * | 40.2 ± 3.9 | 39.7 ± 1.4 | 45.3 ± 1.3 | 41.4 ± 2.1 | 48.0 ± 2.4 |

The results are expressed as nmol/h/mg protein and represent the mean ± S.E.M. of 7 experiments.
* $P < 0.05$ for comparison with the control group.

Table 1). Parallel to the changes observed in the maximal activities of the enzymes, lymphocytes from PNX animals also showed a reduced glucose and glutamine consumption (23% for both substrates) and decarboxylation (64% for glucose and 27% for glutamine; Table 2). These metabolic changes were accompanied by a reduction in the proliferative response of cultured lymphocytes (30%; Table 3).

Lymphocytes from Walker-256 tumour-bearing (TB) rats showed the same pattern of changes as those in cells from PNX animals, presenting a reduced HK (60%), CS (41%) and G6PDh (38%) activities (Table 1). These changes are accompanied by a decrease in glucose consumption (30%) and lactate production (62%) and an increase in glucose decarboxylation (1.9-fold) (Table 2). Concerning the metabolism of glutamine, besides no changes in glutaminase activity, these cells presented an augmented glutamine consumption (33%) and decarboxylation (42%) (Table 2).

The treatment of TB rats with melatonin restored the maximal activities of HK (2.4-fold) and CS (54%) but did not affect G6PDh and glutaminase (GLUT; Table 1). On the other hand, pinealectomized (PNX) TB rats presented the same pattern of changes for HK and CS but showed an increase in GLUT (37%) and no changes in G6PDh in comparison with TB rats (Table 1). The treatment of these animals with a daily dose of melatonin restored the activities of the four enzymes to the levels observed for the control group (Table 1).

Melatonin treatment of TB rats restored glucose consumption, lactate production and glucose decarboxylation to levels similar to the control group (Table 2), did not affect the consumption of glutamine but enhanced the flux of labelled glutamine through the Krebs cycle (Table 2). Lymphocytes from PNX-TB rats showed the same pattern of changes observed for the cells from TB rats, presenting, however, an even greater reduction in glucose consumption and an increase in glutamine consumption (72%; Table 2). In PNX-TB rats treated with melatonin, the lymphocytes presented the same pattern of metabolite utilization as that shown by cells obtained from control rats (Table 2).

These metabolic changes are accompanied by a new profile of cell proliferation. In fact, lymphocytes from TB rats presented an increased proliferative capacity (59%, 28% and 30% for a mixed cell population, ConA and LPS stimulated cells, respectively; Table 3). The treatment with melatonin increased even more the proliferative response of lymphocytes (92%, 49% and 64%, respectively, for cells without stimulation and cultivated in the presence of ConA and LPS, respectively). Pinealectomy abolishes the changes provoked by the presence of the tumour, but did not affect the response of the cells in melatonin PNX-TB rats (Table 3).

As well as for lymphocytes, macrophages from PNX rats presented a series of metabolic changes, including a reduction in the activity of HK, CS and glutaminase (19%,

Table 3
Proliferation index of lymphocytes obtained from tumour-bearing (TB) and pinealectomized tumour-bearing rats (PNX-TB) and control rats (C) treated with a daily dose of melatonin (MEL)

| Ly | C | PNX | PNX-MEL | TB | TB-MEL | PNX-TB | PNX-TB-MEL |
|---|---|---|---|---|---|---|---|
| C | 1032.4 ± 93.8 | 723.1 ± 61.0 * | 1176.2 ± 87.3 | 1644.3 ± 99.3 * | 1984.3 ± 87.5 * | 1232.4 ± 91.3 | 1843.2 ± 142.3 * |
| ConA | 2843.2 ± 144.3 | 1874.3 ± 93.2 * | 2981.8 ± 79.4 | 3644.8 ± 128.7 * | 4241.3 ± 243.4 * | 2994.3 ± 148.7 | 4304.7 ± 150.0 * |
| LPS | 2104.7 ± 94.8 | 1783.2 ± 86.5 * | 2249.3 ± 43.2 | 2740.4 ± 149.0 * | 3442.8 ± 213.6 * | 2004.3 ± 163.2 | 3218.7 ± 214.1 * |

The results are expressed as DPM/$10^5$ cells and represent the mean ± S.E.M. of 7 experiments. The cells were cultivated for 48 h and then pulsed with [6-$^3$H]thymidine. Concanavalin A (ConA) and lipopolysaccharide (LPS) were added to the culture to activate T and B lymphocytes, respectively.
* $P < 0.05$ for comparison with the control group.

Table 4
Maximal activities of hexokinase (HK), citrate synthase (CS), glucose-6-phosphate dehydrogenase (G6PDh) and glutaminase (GLUT) in macrophages obtained from tumour-bearing (TB) and pinealectomized tumour-bearing rats (PNX-TB) and control rats (C) treated with a daily dose of melatonin (MEL)

| MØ | C | PNX | PNX-MEL | TB | TB-MEL | PNX-TB | PNX-TB-MEL |
|---|---|---|---|---|---|---|---|
| HK | 204.3 ± 21.4 | 165.3 ± 14.2* | 211.1 ± 16.3 | 464.3 ± 16.3* | 432.8 ± 24.1* | 531.3 ± 28.4* | 407.3 ± 17.8* |
| CS | 28.3 ± 1.7 | 11.6 ± 1.1* | 27.9 ± 1.1 | 54.1 ± 3.1* | 50.4 ± 2.8* | 47.3 ± 1.9* | 51.2 ± 2.1* |
| G6PDh | 12.1 ± 0.5 | 10.7 ± 1.3 | 14.5 ± 2.0 | 24.0 ± 1.3* | 26.4 ± 0.7* | 31.3 ± 2.4* | 23.4 ± 1.8* |
| GLUT | 117.3 ± 5.3 | 96.3 ± 2.1* | 127.3 ± 6.5 | 156.8 ± 6.4* | 168.3 ± 5.1* | 81.2 ± 3.6* | 153.2 ± 13.1* |

The results are expressed as nmol/min/mg protein and represent the mean ± S.E.M. of 7 experiments.
* $P < 0.05$ for comparison with the control group.

59% and 18%, respectively; Table 4). These cells also showed a reduction in substrate consumption and oxidation (23% for glucose consumption, 27% for its decarboxylation, 30% for glutamine consumption and 26% for its decarboxylation; Table 5). It is interesting to note that, differently for the lymphocytes, macrophages showed the same pattern of glucose compartimentalization, in control and PNX rats, keeping the same proportion of glucose deviated to the Krebs cycle and those found as lactate (Table 5). Lymphocytes form PNX rats, on the other hand, presented an increased diversion of glucose to lactate when compared to cells from normal rats (89% and 75%, respectively for PNX and normal rats; Table 2).

Macrophages obtained from TB rats showed an increase in the activities of the four enzymes assayed (2.27-fold, 91%, 98% and 33% for HK, CS, G6PDh and GLUT, respectively; Table 4). The treatment of the rats with melatonin did not affect the enzyme changes profile. Cells obtained from PNX-TB rats, however, presented increased HK (15%) and G6PDh (30%) activities and a reduced activity of CS (12%) and GLUT (48%) in comparison with cells from TB rats (Table 4). The treatment of the rats from this group with melatonin, however, restored the same pattern of enzyme activities shown by macrophages from TB rats (Table 4).

Macrophages from TB rats presented an augmented consumption of glucose (31%) that is accompanied by a small increase in lactate production (14%) and a much more pronounced flux of glucose through the Krebs cycle (50%; Table 5). The consumption and decarboxylation of glutamine were also augmented in macrophages from TB rats (71% and 55%, respectively; Table 5). The treatment of these rats with melatonin slightly changes the metabolic profile of glucose, diminishing lactate production (11%) and increasing the flux of glucose to the Krebs cycle (75%; Table 5). The consumption of glutamine and its decarboxylation were also improved (20% and 92%, respectively; Table 5). The presence of the tumour in PNX rats provoked an enhancement of glucose metabolism, increasing its consumption (25%), decarboxylation (2.1-fold) and its conversion to lactate (34%) in comparison with cells obtained from TB rats (Table 5). Macrophages from PNX-TB rats, however, presented a decreased glutamine consumption (53%) and flux through the Krebs cycle (60%) in comparison with macrophages obtained from TB rats (Table 5). The treatment of these animals with melatonin enhanced the diversion of glucose and glutamine through the tricarboxylic acid cycle (66% and 45%, respectively) in comparison with cells from TB rats (Table 5).

The functional parameters evaluated for macrophages, hydrogen peroxide production and phagocytosis, were augmented by the presence of the tumour (5.2- and 2.4-fold, respectively; Table 6). These changes, however, were not affected for any of the other experimental conditions, except for a small increase (20%) in hydrogen peroxide

Table 5
Glucose and glutamine consumption (C-GLUC and C-GLUT, respectively) and their decarboxylation rate (D-GLUC and D-GLUT) and lactate production in macrophages obtained from tumour-bearing (TB) and pinealectomized tumour-bearing rats (PNX-TB) and control rats (C) treated with a daily dose of melatonin (MEL)

| MØ | C | PNX | PNX-MEL | TB | TB-MEL | PNX-TB | PNX-TB-MEL |
|---|---|---|---|---|---|---|---|
| C GLUC | 186.4 ± 9.4 | 143.0 ± 6.2* | 193.2 ± 8.3 | 244.3 ± 10.4* | 251.0 ± 12.1* | 307.2 ± 10.4* | 240.4 ± 12.1* |
| LACT | 302.1 ± 14.3 | 249.5 ± 8.6* | 299.1 ± 5.5 | 344.3 ± 12.1* | 305.4 ± 14.1 | 464.3 ± 9.5* | 294.3 ± 24.7 |
| D-GLUC | 23.0 ± 2.1 | 16.7 ± 1.1* | 24.1 ± 1.1 | 34.3 ± 1.7* | 60.1 ± 4.2* | 72.3 ± 2.7* | 57.1 ± 2.4* |
| C-GLUT | 45.6 ± 2.1 | 32.1 ± 1.8* | 43.0 ± 2.0 | 78.4 ± 2.4* | 94.3 ± 4.8* | 36.3 ± 3.0* | 74.8 ± 2.3* |
| D-GLUT | 18.3 ± 0.3 | 13.5 ± 0.6* | 19.4 ± 1.1 | 28.5 ± 1.2* | 54.8 ± 2.6* | 11.4 ± 0.9* | 41.5 ± 0.8* |

The results are expressed as nmol/h/mg protein and represent the mean ± S.E.M. of 7 experiments.
* $P < 0.05$ for comparison with the control group.

Table 6
Hydrogen peroxide production and the percentage of zymosan particles phagocytosis (PI) from macrophages obtained from tumour-bearing (TB) and pinealectomized tumour-bearing rats (PNX-TB) and control rats (C) treated with a daily dose of melatonin (MEL)

| MØ | C | PNX | PNX-MEL | TB | TB-MEL | PNX-TB | PNX-TB-MEL |
|---|---|---|---|---|---|---|---|
| $H_2O_2$ | 9.8 ± 0.4 | 11.3 ± 2.1 | 11.9 ± 1.1 | 51.3 ± 2.7 * | 58.3 ± 3.4 * | 62.3 ± 4.4 * | 63.3 ± 5.0 * |
| PI | 18.6 ± 1.7 | 12.8 ± 0.5 * | 16.9 ± 0.5 | 45.3 ± 2.5 * | 51.3 ± 3.8 * | 52.4 ± 2.7 * | 49.3 ± 1.7 * |

The results are expressed as nmol/h/mg protein for hydrogen peroxide production and represent the mean ± S.E.M. of 7 experiments.
* $P < 0.05$ for comparison with the control group.

production observed in both groups of PNX rats in comparison with the production observed for cells obtained from TB rats (Table 6).

Melatonin injected in normal rats, however, did not modify the metabolic profile of macrophages and lymphocytes, as could be seen in all the Tables.

## 4. Discussion

The modulation of the immune system through neuropeptides and neurohormones contributes to the assumption of the existence of a neuro-endocrine-immune axis (Maestroni et al., 1987a,b, 1988a,b). Some enkephalins, endorphins and endogenous opioid peptides alter NK cytotoxicity, lymphokine and antibody production by T and B lymphocytes and the response of macrophages to interferon-$\gamma$, adrenaline and to the presence of tumours in the organism (Blask, 1984; Niles, 1989). Indolamines seem to be part of an immunorregulatory circuit. Interferon-$\gamma$ stimulates the production of serotonin and melatonin by macrophages and lymphocytes, whereas these indolamines are capable of altering the synthesis of interferon-$\gamma$ (Finocchiaro et al., 1991; Maestroni et al., 1996). Melatonin (*N*-acetyl-5-methoxytryptamine), a hormone produced by the pineal gland, in particular plays an important role in the immune system, modulating NK cytotoxicity, lymphocyte proliferative response to mitogen and interferon-$\gamma$ production (Del Gobbo et al., 1989; Maestroni et al., 1996). These effects of melatonin upon immune cell functions could be, at least in part, related to the presence of specific receptors in such cells, as reported by Calvo et al. (1995), Maestroni (1995), Rafii-El-Idrissi et al. (1995) and Maestroni et al. (1996). The mechanisms involved in intracellular signalling are, however, still under investigation by several groups.

Lymphocytes, as highly proliferative cells, and macrophages presenting a high secretory and phagocytic activity, need great amounts of energy and substrates for the synthesis of macromolecules. To cope with these functions, both cell types consume high amounts of glucose and glutamine. The inability to metabolise glucose (important source of molecules for synthetic pathways) and/or glutamine (the main source of energy for these cell types, and also a source of molecules for cell division process) impairs lymphocyte and macrophage function (Curi and Newsholme, 1989; Costa Rosa et al., 1993, 1996a,b). In the presence of the Walker-256 tumour, lymphocytes and macrophages presented metabolic changes concerning glucose and glutamine utilisation. These changes are related to a decrease lymphocyte proliferative response and a reduced production of hydrogen peroxide and phagocytosis by macrophages.

Lymphocytes from Walker-256 tumour-bearing rats presented a decrease in the maximal activity of hexokinase, citrate synthase and glucose-6-phosphate dehydrogenase. Together with these changes there was an increase in the flux of glucose through the Krebs cycle. In fact, in lymphocytes from control rats, 18% of glucose was decarboxylated, while in lymphocytes from TB rats, 49% of the consumed glucose was metabolized through the Krebs cycle, although there was a decrease in glucose consumption. On the other hand there was a small increase in glutamine consumption (33%) and a reduction in the oxidation of this substrate (10%). It is interesting to note that lymphocytes from TB rats show a preference for utilising certain substrates for oxidation, in agreement with previous findings of Seelaender et al. (1996), who showed that lymphocytes from TB rats oxidized 17-fold more long chain fatty acids when cultivated in the presence of homologous serum than those obtained from control rats. These changes in the metabolic profile of lymphocytes, with the prevalence of oxidative processes, could provoke a reduction in their synthetic capacity, thus interfering in the production of cytokines and other substances important to cell function (Curi and Newsholme, 1989).

The treatment of the animals with melatonin partially restored the metabolic response of lymphocytes to the presence of the tumour. In fact, only the activity of G6PDh was not changed by the treatment. The flux of glutamine through the Krebs cycle was enhanced (from 48% to 66%). These cells presented a higher proliferative response to ConA and LPS than those from TB rats, suggesting more efficiency in the response of the immune system to the presence of the tumour.

Another important cell type greatly involved in the host's response to the presence of tumours is the macrophage (Adams and Hamilton, 1992; Auger and Ross, 1992). Differently from lymphocytes, macrophages from TB rats presented an increase in the maximal activities of the enzymes studied. Such changes were accompanied by an increase in the consumption of glucose and glutamine, as well as in the decarboxylation of these substrates. No

changes in the profile of metabolites formed from glucose and glutamine was observed, however, in these cells. Therefore, the increase in the consumption of the studied substrates followed the same pattern of metabolization.

The results concerning functional aspects of macrophages demonstrated increased hydrogen peroxide production and phagocytic activity, suggesting that the cells are stimulated by the presence of the tumour. The cells from rats treated with melatonin showed the same metabolic profile, except for the consumption and oxidation of glutamine that were increased by 20% and 92%, respectively, as well as the oxidation of glucose (increased by 75%). Nevertheless, these changes were not enough to amplify the modification in either hydrogen peroxide production nor phagocytosis, but could be involved in the high energy-consuming production of other immune response-related substances by the macrophages, such as cytokines, prostaglandins, and free radicals.

To certify that melatonin is an important mediator of the changes observed, we have carried out the same experiments with lymphocytes and macrophages from pinealectomized (PNX) rats. The metabolic response of cells from TB-PNX rats was almost the same as those observed for TB rats, except for an increase of 40% in glutaminase in the lymphocytes coupled with an increased consumption of glutamine (37%), which was, however, not diverted through the Krebs cycle. It is interesting to note that the proliferative response of the cells was similar to that from control rats. These data suggest that the changes in glutamine metabolism observed in PNX-TB rats impaired the ability of lymphocytes to proliferate under the stimulus of the presence of the tumour.

Macrophages from PNX-TB rats presented, as did lymphocytes, the same pattern of changes in the maximal enzyme activities as those verified for the cells from TB rats, except for the decreased glutamine consumption and decarboxylation observed for the latter. These changes did not modify the production of hydrogen peroxide and phagocytosis by the cells. It is interesting to note, however, that PNX-rats are more susceptible to death induced by the Walker-256 tumour implantation. The treatment of TB rats with a daily dose of melatonin, at the onset of the dark period increases the animal survival span in 84.6% (TB rats died $13.0 \pm 0.58$ days after tumour implantation, while those treated with the hormone in $24.0 \pm 0.71$ days). The same pattern of response was observed in PNX tumour-bearing rats treated with melatonin, who presented a 2.07-fold increase in life expectancy in relation to the TB group.

Our results strongly suggest an important role for the immune cells as mediators of melatonin action in rats bearing the Walker-256 tumour, as this hormone reverted the metabolic changes and recovered the functional response of lymphocytes and macrophages, and the maintenance of the metabolic profile of these cells by the hormone seems to be crucial for the development of a proper immune response to tumour implantation.

The hypothesis that the action of melatonin could also be exerted directly upon the tumour, altering tumour cell metabolism (Blask et al., 1997; Sauer et al., 1997) cannot be discarded. The possible direct effect of melatonin upon Walker-256 tumour cell is now under investigation in our laboratory.

## References


Adams, D.O., Hamilton, T.A., 1992. Molecular basis of macrophage activation, diversity and its origins. In: Lewis, C.E., McGee, J.O'D. (Eds.), The natural immune system. The macrophage. IRL Press, Oxford, pp. 75–114.

Almeida, A.F., Curi, R., Newsholme, P., Newsholme, E.A., 1989. Maximal activities of key enzymes of glutaminolysis, glycolysis, Krebs cycle and pentose-phosphate pathway of several tissues in mature and aged rats. Int. J. Biochem. 21, 937–940.

Artz, E., Fernández-Castelo, S., Finocchiaro, L.M.E., Criscuolo, M., Díaz, A., Finkielman, S., Nahmod, V.E., 1988. J. Clin. Immunol. 8, 513–520.

Auger, M.J., Ross, J.A., 1992. The biology of the macrophage. In: Lewis, C.E., McGee, J.O'D. (Eds.), The natural immune system. The macrophage. IRL Press, Oxford, pp. 1–74.

Bennet, J.P., 1985. In: Yamamura, H.I., Enna, S.J., Kuhar, M.J. (Eds.), Neurotransmitter Receptor Binding, Raven Press, New York, pp. 57–90.

Bergmeyer, H.U., Bernt, E., Schmidt, F., Stork, H., 1974. D-glucose. Determination with kexokinase and glucose-6-phosphate dehydrogenase. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis, Vol. 3, pp. 1196–1201,1 Academic Press, New York, pp. 1196–1201.

Blalock, J.E., 1992. Production of peptide hormones and neurotransmitters by the immune system. In: Blalock, J.E. (Ed.), Neuroimmunoendocrinology, Chemical Immunology, Vol. 52, Karger, Basel, pp. 1–19.

Blalock, J.E., 1994a. The immune system, our sixth sense. Immunologist 2, 8–15.

Blalock, J.E., 1994b. The syntax of immune neuroendocrine communication. Immunol. Today 15, 504–511.

Blask, D.E., 1984. In: Reiter, R.J. (Ed.), The Pineal Gland, Raven Press, New York, pp. 253–284.

Blask, D.E., Wilson, S.T., Zalatan, F.T.I., 1997. Physiological melatonin inhibition of human breast cancer cell growth in vitro, evidence for a glutathione-mediated pathway. Cancer Res. 57, 1909–1914.

Calvo, J.R., Rafii-El-Idrissi, M., Pozo, D., Guerrero, J.M., 1995. Immunomodulatory role of melatonin, specific binding sites in human and rodent lymphoid cells. J. Pineal Res. 18, 119–126.

Cardinali, J.P., Vacas, M.I., Boyer, E.E., 1979. Specific binding of melatonin in bovine brain. J. Endocrinol. 105, 437–441.

Cooney, G., Newsholme, E.A., 1982. Does brown adipose tissue have a metabolic role in rat?. TIBS 9, 303–305.

Cooney, G., Taegmeyer, H., Newsholme, E.A., 1981. Tricarboxylic acid cycle flux and enzyme activities in the isolated working rat heart. Biochem. J. 200, 701–703.

Costa Rosa, L.F.B.P., Safi, D.A., Guimarães, A.R.P., 1996a. The effect of n-3 PUFA rich diet upon macrophage and lymphocyte metabolism and function. Biochem. Mol. Biol. Int. 40, 833–842.

Costa Rosa, L.F.B.P., Safi, D.A., Cury, Y., Curi, R., 1993. Effect of epinephrine on glucose metabolism and hydrogen peroxide content in incubated rat macrophages. Biochem. Pharmacol. 63, 2235–2241.

Costa Rosa, L.F.B.P., Safi, D.A., Cury, Y., Curi, R., 1996b. Effect of insulin on macrophage metabolism and function. Cell. Biochem. Function 14, 33–43.

Crabtree, B., Leech, A.R., Newsholme, E.A., 1979. Measurement of enzyme activities in crude extracts of tissues. In: Pogson, C. (Ed.),

Techniques in the Life Sciences — Biochemistry. Techniques in Metabolic Research — Part I, Vol. B211, Elsevier/North Holland, Amsterdam, pp. 1–37.

Curi, R., Newsholme, E.A., 1989. The effect of adenine nucleotides on the rate and fate of glutamine utilization by icubated mitochondria isolated from rat mesenteric lymph nodes. Mol. Cell. Biochem. 86, 71–76.

Curi, R., Newsholme, P., Newsholme, E.A., 1986. Intracellular distribution of some enzymes of the glutamine utilisation pathway in rat lymphocytes. Biochem. Biophys. Res. Commun. 138, 318–322.

Curi, R., Newsholme, P., Newsholme, E.A., 1988. Metabolism of pyruvate by isolated rat mesenteric lymphocytes, lymphocyte mitochondria and isolated mouse macrophages. Biochem. J. 250, 383–388.

Curi, R., Williams, J.F., Newsholme, E.A., 1989. Pyruvate metabolism by lymphocytes, evidence for an additional ketogenic tissue. Biochem. Int. 19, 755–767.

Del Gobbo, V., Libri, V., Villani, N., Calib, R., Nistico, G., 1989. Pinealectomy inhibits interleukin-2 production and natural killer activity in mice. Int. J. Immunopharmacol. 11, 567–577.

Engle, P.C., Jones, J.B., 1978. Causes and elimination of erratic blanks in enzymatic metabolite assays involving the use of NAD in alkaline hydrazine buffers, Improved conditions for the assay of L-glutamate, L-lactate and other metabolites. Anal. Biochem. 88, 475–484.

Finocchiaro, L.M.E., Nahmod, V.E., Launay, J.M., 1991. Melatonin biosynthesis and metabolism in peripheral blood mononuclear leucocytes. Biochem. J. 280, 727–731.

Hellstrand, K., Hermodsson, S., 1987. Role of serotonin in the regulation of human natural killer cell cytotoxicity. J. Immunol. 139, 869–875.

Hellstrand, K., Hermodsson, S., 1990. Monocyte-mediated suppression of lL-2 induced NK-cell activation. Regulation by 5-HTLA-type serotonin receptors. Scand. J. Imunol. 32, 183–192.

Hoffman, R.A., Reiter, R.J., 1965. Rapid pinealectomy in hamsters and other small rodents. Anat. Rec. 153, 19–22.

Jackson, J.C., Cross, R.J., Walker, R.F., Markesberry, W.R., Brooks, W.H., Roszman, T.L., 1985. Immunology 54, 505–512.

Kowalchuk, J.M., Curi, R., Newsholme, E.A., 1988. Glutamine metabolism in isolated incubated adipocytes of the rat. Biochem. J. 249, 705–708.

Lissoni, P., Barni, S., Tancini, G., Rovelli, F., Ardizzoia, A., Conti, A., Maestroni, G.J.M., 1993. A study of the mechanisms involved in the immunostimulatory action of the pineal hormone in cancer patients. Oncology 50, 399–402.

Maestroni, G.J.M., Conti, A., 1991. Action of melatonin on immune system. In: Fraschini, F., Reiter, R.J. (Eds.), Role of melatonin and pineal peptides in neuroimmunomodulation. Plenum Press, New York, pp. 201–210.

Maestroni, G.J.M., 1995. T-helper-2 lymphocytes as a peripheral target of melatonin. J. Pineal Res. 18, 84–89.

Maestroni, G.J.M., Conti, A., Pierpaoli, W., 1987a. Role of the pineal gland in immunity. II. Melatonin enhances the antibody response via an opiatergic mechanism. Clin. Exp. Immunol. 68, 384–391.

Maestroni, G.J.M., Conti, A., Pierpaoli, W., 1987b. The pineal gland and the circadian, opiatergic, immunoregulatory role of melatonin. Ann. NY Acad. Sci. 496, 67–77.

Maestroni, G.J.M., Conti, A., Pierpaoli, W., 1988a. Pineal melatonin, its fundamental immunorregulatory role in aging and cancer. Ann. NY Acad. Sci. 521, 140–148.

Maestroni, G.J.M., Conti, A., Pierpaoli, W., 1988b. Role of the pineal gland in immunity. III. Melatonin antagonizes the immunosuppressive efect of acute stress via an opiatergic mechanism. Immunol. 63, 465–469.

Maestroni, G.J.M., Conti, A., Lissoni, P., Archilli, C., Cattaneo, G., Tancini, G., Barni, S., 1990. Neuroendocrine strategy with pineal hormone melatonin to enhance the antitumour activity of interleukin-2. Eur. J. Cancer 26, 194.

Maestroni, G.J.M., Hertens, E., Galli, P., Conti, A., Pedrinis, E., 1996. Melatonin-induced T-helper cell hematopoietic cytokines resembling both interleukin-4 and dynorphin. J. Pineal Res. 21, 131–139.

Newsholme, E.A., Newsholme, P., Curi, R., Crabtree, B., Ardawi, M.S.M., 1989. Glutamine metabolism in different tissues. In: Kinney, J.M., Borum, P.G. (Eds.), Perspectives in clinical nutrition, Urban and Scharzenberg, Baltimore, USA, pp. 71–98.

Newsholme, P., Gordon, S., Newsholme, E.A., 1987. Rates of utilization and fates of glucose, glutamine, pyruvate, fatty acids and ketone bodies by mouse macrophages. Biochem. J. 242, 631–636.

Niles, L.P., 1989. High-affinity binding for melatonin in hamster spleen. Med. Sci. Res. 17, 179–180.

Panerai, A.E., Bianchi, M., Locatelli, L., Ciciliato, I., Rubboli, F., Sacerdote, P., 1991. Opioids in immune cells. In: Fraschini, F., Reiter, R.J. (Eds.), Role of melatonin and pineal peptides in neuroimmunomodulation. Plenum Press, New York, pp. 211–217.

Pick, E., Mizel, D., 1981. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. J. Immunol. Methods 46, 211–226.

Poon, A.M.S., Pang, S.F., 1992. 2-[$^{125}$I]Iodomelatonin binding sites in spleens of guinea pigs. Life Sciences 50, 1719–1726.

Rafii-El-Idrissi, M., Calvo, J.R., Pozo, D., Harmouch, A., Guerrero, J.M., 1995. Specific binding of 2-[$^{125}$I]iodomelatonin by rat splenocytes, characterization and its role on regulation of cyclic AMP production. J. Neuroimmunol. 57, 171–178.

Regelson, W., Pierpaoli, W., 1987. Melatonin. A rediscovered antitumour hormone? Its relation to surface receptors, sex, steroid metabolism, immunologic response and chronobiological factors in tumour growth and therapy. Cancer Invest. 5, 379–385.

Sauer, L.A., Dauchy, R.T., Blask, D.E., 1997. A novel mechanism for the regulation of tumour growth in vivo. Physiological concentration of melatonin inhibit fatty acid uptake in hepatoma 7288CTC perfused in situ. Cancer Res. 37, 224, abstract.

Seelaender, M.C.L., Carmella, M.C., Werner, M., Curi, R., 1996. Hormonal alterations in Walker-256 tumour-bearing rats. Possible role of calcium for the maintenance of cachexia. Cancer Res. Ther. Control 5, 29–33.

Stanley, J.C., Newsholme, E.A., 1985. The effect of dietary guar gum on the activities of some key enzymes of the carbohydrate and lipid metabolism in mouse liver. Br. J. Nutrition 53, 215–222.

Vieira, R., Nascimento, R., Arizawa, S., Curi, R., 1990. Development of equipment for lymphocyte isolation and culture. Arq. Biol. Tecnol. 33, 819–829.

Weigent, D.A., Blalock, J.E., 1995. Associations between the neuroendocrine and immune systems. J. Leukoc. Biol. 57, 137–150.

Windmueller, H.G., Spaeth, A.E., 1974. Uptake and metabolism of plasma glutamine by the small intestine. J. Biol. Chem. 249, 5070–5079.