ANTITUMOR AND IMMUNOMODULATORY EFFECTS OF PINEAL INDOLES

by

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Contents

Abstract

The cytotoxic actions of pineal indoles were studied on different tumor cell lines including mouse melanoma cell line (B16), human choriocarcinoma cell line (JAr), sarcoma cell line (S180), macrophage-like cell line (PU5) and fibroblast cell line (3T3). The pineal indoles examined included melatonin, methoxytryptamine, methoxytryptophol, hydroxytryptophol, serotonin, hydroxyindoleacetic acid and methoxyindoleacetic acid. It was found that methoxytryptamine inhibited incorporation of ^{3}H-thymidine, ^{3}H-uridine, and ^{3}H-leucine into mouse melanoma cells with the highest potency. ^{3}H-Thymidine incorporation into sarcoma and PU5 cells was inhibited by methoxytryptamine with the highest potency. Methoxytryptamine exerted the most potent inhibitory effect on crystal violet uptake by fibroblasts. Serotonin and hydroxyindoleacetic acid exhibited low inhibitory potencies in the aforementioned cell lines. Neither melatonin nor methoxytryptamine increased the release of glutamate-oxaloacetate transaminase by cultured rat hepatocytes into the culture medium although there was a slight enhancement of release of glutamate pyruvate transaminase and a slight inhibition of crystal violet uptake by the hepatocytes. The results indicate that various pineal indoles exerted cytotoxic actions against tumor cell lines but had little or no effect on the viability of hepatocytes.

Both in vivo and in vitro treatments with melatonin and methoxytryptamine produced an increase in nitrite production by peritoneal macrophages, suggesting that the pineal indoles exert both direct cytotoxic actions on tumor cells as well as indirect antitumor effects by enhancing the tumoricidal activity of macrophages. Treatment with

methoxytryptamine under in vivo and in vitro conditions caused peritoneal macrophages to generate less hydrogen peroxide, but similar treatment with melatonin did not produce a marked alteration in hydrogen peroxide production. Both in vivo and in vitro treatment with melatonin led to an increased superoxide production by peritoneal macrophages. On the other hand, in vitro methoxytryptamine treatment produced an increase in superoxide but in vivo methoxytryptamine treatment produced just the opposite. The results suggest that superoxide and hydrogen peroxide contribute little to the enhancement of tumoricidal activity of peritoneal macrophages by pineal indoles. In vivo treatment with melatonin and methoxytryptamine led to an increase in production of tumor necrosis factor-α and its mRNA by peritoneal macrophages, while in vitro methoxytryptamine decreased and in vitro melatonin treatment did not have any effect on production of tumor necrosis factor-α. The results suggest that the secretion of hydrogen peroxide and the secretion of nitrite involve two independent pathways and that there are some differences between the immunomodulatory actions of melatonin and methoxytryptamine. Some of the discrepancies between the actions of pineal indoles in vivo and in vitro may be attributed to the fact that in the in vivo study the macrophages had been primed or stimulated by the pineal indole for a longer duration and they subsequently demonstrated a greater response to the stimulant in vitro. The ability of melatonin and methoxytryptamine to augment production of nitrite, and tumor necrosis factor, which are molecules involved in the combat against tumor cells, is consistent with the in vitro cytotoxic actions of the pineal indoles.

When compared to splenocytes from control mice, splenocytes from melatonin-treated

mice exhibited greater mitogenic responses to concanavalin A and lipopolysaccharide while splenocytes from methoxytryptamine-treated mice manifested a lower mitogenic response to concanavalin A and a greater mitogenic response to lipopolysaccharide. Splenocytes from both melatonin-treated and methoxytryptamine-treated mice produced more γ-interferon and interleukin-2 than splenocytes from control mice, with melatonin exerting a stronger stimulatory effect. Crude lymphokines from splenocytes of melatonin -treated and methoxytryptamine-treated mice were able to stimulate nitrite production by peritoneal macrophages (from control mice, melatonin-treated mice, as well as from methoxytryptamine-treated mice) to a greater extent than crude lymphokines from splenocytes of control mice, suggesting that macrophage activating factor was present at higher concentrations in preparations of crude lymphokines from splenocytes of melatonin-treated and methoxytryptamine-treated mice than crude lymphokine preparation from control mice. Crude lymphokines from melatonin-treated mice had a greater stimulatory effect on macrophage nitrite production, implying that melatonin exerted a greater stimulatory effect on lymphocytes than methoxytryptamine. Macrophages from melatonin-treated and methoxytryptamine-treated mice produced more nitrite than macrophages from control mice when they were activated by crude lymphokines from melatonin-treated mice or methoxytryptamine-treated mice, suggesting that macrophages from melatonin-treated and methoxytryptamine-treated mice were more sensitive to the action of lymphokines than macrophages from control mice. Macrophages from methoxytryptamine-treated mice produced the highest nitrite level indicating that methoxytryptamine treatment exerted a stronger stimulatory effect on macrophages than melatonin. Crude lymphokines from melatonin-treated mice but not those from

methoxytryptamine-treated mice were able to stimulate greater inhibition of the growth of murine mastocytoma P815 cells by macrophages from control mice, melatonin-treated mice and methoxytryptamine-treated mice when compared to crude lymphokines from control mice, indicating that macrophage activating factor was present at a higher concentration by crude lymphokines from melatonin-treated mice and that melatonin exerts a stronger stimulatory effect on lymphocytes than methoxytryptamine. Macrophages from both melatonin-treated and methoxytryptamine-treated mice elicited greater inhibition of the growth of P815 cells than macrophages from control mice when they were stimulated by crude lymphokines from melatonin-treated mice, suggesting that macrophages from melatonin-treated and methoxytryptamine-treated mice were more sensitive than macrophages from control mice to crude lymphokine preparation from melatonin-treated mice. Again the higher response observed in macrophages from methoxytryptamine-treated mice suggest that methoxytryptamine exerts a stronger stimulatory effect on macrophages than melatonin. The lack of differential response among macrophages of the various groups of mice to crude lymphokines from control mice and methoxytryptamine-treated mice may be attributed to the lower activity of macrophages activating factor in these lymphokine preparations.

Results of this study revealed a cytotoxic action of pineal indoles on tumor cell lines and an immunostimulatory action on macrophages and splenocytes.

Chapter 1 General Introduction

1.1 The pineal gland

The human pineal gland, also called epiphysis cerebri, is located near the third ventricle of the brain. It measures about 5 - 8 mm in length and 5 mm in width, weighs about 2 g and is coated with a soft membrane. Blood from the heart enters the pineal gland via the posterior cerebral arteries and leaves it via the great cerebral veins (Williams & Warwick 1980).

1.2 Discovery of melatonin

Melatonin and its derivatives were studied in this project. Melatonin was first isolated in 1958 by Lerner et al. They found that melatonin inhibited pigment cells and suggested that melatonin would inhibit melanoma (Lerner 1958).

1.3 Synthesis of melatonin

In the pinealocytes, tryptophan is hydroxylated by tryptophan hydroxylase to form 5-hydroxytryptophan (Lovenberg 1967). 5-Hydroxytryptophan is then decarboxylated by L-aromatic amino acid decarboxylase to form 5-hydroxytryptamine, i.e. serotonin (Lovenberg 1962). Serotonin in the pineal gland has several metabolic fates. First, it may be converted into melatonin by the action of 2 enzymes, N-acetyltransferase and hydroxyindole-O-methyltransferase. Second, it may undergo O-methylation to

form 5-methoxytryptamine by hydroxyindole-O-methyltransferase (Axelrod 1961). Third, it may undergo oxidative deamination to form 5-hydroxyindole acetaldehyde with the help of monoamine oxidase (Keglevic 1968). 5-Hydroxyindole acetaldehyde can then be oxidized to form 5-hydroxyindole acetic acid and 5-hydroxytryptophol. 5-Hydroxyindoleacetic acid and 5-hydroxytryptophol may undergo O-methylation to form 5-methoxyindoleacetic acid and 5-methoxytryptophol respectively by hydroxyindole-O-methyltransferase (Miles 1988). Because of their pineal origin and possession of an indole nucleus in their molecules, melatonin and its derivatives are named pineal indoles.

1.4 Physiology of melatonin and its derivatives

The pineal gland is known as a neurochemical transducer (Axelrod 1974). It seems to translate photoperiodic information into chemical message. Melatonin is believed to be the chemical messenger because of its circadian changes in serum level (Josephine 1988). Its blood concentration peaks at night and is maintained low in the daytime (Reiter 1991). Nocturnal serum melatonin concentration in healthy young men was about 100 pg/ml while that in old men was about 20 pg/ml.

Different hamster and rat organs have been shown to have cytoplasmic melatonin receptors. The presence of melatonin receptor was demonstrated by using the agonist, 2-[^{125}I] iodomelatonin and the competitive antagonist, luzindole (Margarita 1988). The ovary, uterus, liver, eye, thyroid, and testis showed different melatonin binding with KD values around 250 fmol per mg protein (Michael 1978). The

function of the pineal as a transducer is especially important to photoperiod-sensitive mammals which are mostly seasonal breeders. In these mammals the reproductive potential of the gonad fluctuates with circadian changes in photoperiod and thus with the melatonin level (Reiter 1980). Pineal indoles have been shown to inhibit steroidogenesis in isolated rat Leydig cells as a result of inhibition mostly on 17-20 desmolase (Ng 1987, Ng 1988, Ooi 1989). The number of graafian follicles and corpora lutea and the thickness of uterine myometrium and endometrium were also reduced in female hamsters treated with pineal indoles (Ooi 1989). Both evidences show an inhibitory effect of pineal indoles on the reproductive system. Lennart (1976) tested the melatonin concentration in serum samples collected early in the morning during the menstrual cycle of 5 healthy women and found that the highest melatonin level occurred at the time of menstrual bleeding and that the lowest concentration accompanied the peak of luteinizing hormone. He suggested that low melatonin concentration at the mid-cycle was a permissive factor for ovulation. Chik (1985) also found that morning luteinizing hormone level was elevated in pinealectomized patients. Besides, melatonin may regulate several neuroendocrine functions such as sleep and pituitary hormone secretion in humans (Margarita 1988).

1.5 <u>In vitro</u> tumor biology of melatonin and its derivatives

Blask & Hill have written an extensive review on the antitumor aspects of melatonin (Blask 1988). Blask suggested that the pineal is an oncostatic gland with melatonin playing a major oncostatic role. The effect of melatonin on cell growth, especially that of tumor cells, has been widely studied in the last decade. Some of the results

suggest the role of melatonin as an oncostatic convoy. Melatonin seems to be more effective on hormone-responsive tumors.

Banerjee (1972) reported that melatonin inhibited cell division in protozoans. Margulis (1973) found that melatonin inhibited cell division in onion root tips. In the prepubertal rat, pinealectomy prevented the inhibitory effect of light deprivation on the proliferation of anterior pituitary cells (Blask 1984).

Boucek & Alvarez (1970) studied the effect of serotonin & N-acetylserotonin, the precursors of melatonin, on 5 different cell lines. Serotonin was found to stimulate 3T6 & human embryonic lung fibroblasts while N-acetylserotonin was found to inhibit cell proliferation by 30 %. Neither of them at 10^{-7} M had any effect on Hela cells (human cervical carcinoma), KB cells (human oral epidermoid carcinoma) or BHK cells (golden hamster kidney). Hill (1986) reported that melatonin, N-acetylserotonin and serotonin did not inhibit proliferation of mouse 3T3 and 3T6 and human foreskin fibroblast cell lines (Hill 1986).

Walker (1978) detected a biphasic effect of melatonin on the cloned melanoma cell line B7 obtained from spontaneously arising tumors in a male golden hamster. Melatonin at millimolar concentrations showed 25 % inhibition of ^{3}H-thymidine uptake but melatonin at micromolar concentrations showed 37 % stimulation. There are discrepancies when these results are compared to those of Bartsch. Bartsch (1984) observed that melatonin elicited 60 % inhibition at micromolar concentrations but produced a moderate stimulation of ^{3}H-thymidine uptake by human melanoma

cells at a 10 - fold higher concentration.

Much work has been done using the human breast cancer cell line MCF-7 which possess both estrogen & prolactin receptors. Melatonin at a concentration of 10^{-11} M - 10^{-9} M, which is within the physiological range in human blood, caused 75 % inhibition of cell proliferation in culture, while other indoles such as serotonin, N-acetylserotonin, 5-methoxytryptophol, 5-methoxytryptamine in the same dose range did not have any effects on proliferation of MCF-7 cells (Hill 1986). Osborne (1985) tested melatonin and tamoxifen, a non-steroidal antiestrogen, on MCF-7 cells. He found that nanomolar concentrations of melatonin were more effective than micromolar concentrations of tamoxifen in inhibiting the growth of MCF-7 cells. However, combined treatment with melatonin and tamoxifen produced a degree of growth suppression similar to that of tamoxifen alone. Since tamoxifen was found to block the antiproliferative effects of melatonin and known to bind to estrogen receptors, he postulated that melatonin exerted antiproliferative effects on MCF-7 cells through an interaction with estradiol receptors. Vignon and Rochefort (1985) observed that melatonin inhibited the release of the estradiol-inducible 52 K glycoprotein which stimulated the growth of MCF-7 cells in an autocrine fashion. Blask and Hill (1986) found that melatonin at 10^{-9} M did not have any inhibitory effect but melatonin at 10^{-6} M caused 40 % inhibition of the growth of the estrogen receptor-positive human endometrial cancer cell line RL95. They also found that melatonin was completely ineffective as a growth inhibitor of the human breast cancer cell line BT20 which was devoid of estrogen receptors and hence estrogen - unresponsive. Danforth (1983) found that melatonin increased the estrogen receptor

binding activity of MCF-7 cells. He projected melatonin as a factor to be considered for use in treatment since patients with hormone-dependent (ER-positive) breast cancer have a significantly higher response to hormonal manipulation and a better prognosis.

When tested on the human ovarian carcinoma cell lines SK-OV-3 and JA-1 and the bladder carcinoma cell line RT112, melatonin and N-acetylserotonin exerted a significant growth inhibitory effect in vitro only at relatively high concentrations (0.5 - 1 mg/ml) while 5-methoxytryptamine and 6-hydroxymelatonin were 5 times more effective (Shellard 1989).

1.6 In vivo tumor biology of melatonin

Different animals bearing different tumors were either pinealectomized or melatonin-treated to study the effects of melatonin on the tumors and the survival of the animals. Secretion of melatonin in the animal was prevented by pinealectomy or subjection to a long photoperiod. Lapin (1976) and Blask (1984) observed that pinealectomy in mice bearing Erhlich's tumors enhanced tumor growth. Bartsch (1981) found that melatonin injections in the late afternoon suppressed the growth of Erhlich's tumor significantly in mice subjected to a long photoperiod. Lapin (1981) found that pinealectomy in rats bearing Yoshida tumors stimulated tumor growth while melatonin administration prevented the stimulation of tumor growth and thus enhanced the survival time of the rats. It is another evidence of melatonin being an oncostatic convoy of the animal.

Barone (1972) observed that pinealectomy in rats markedly enhanced the growth of fibrosarcomas. Bartsch (1981) found that melatonin administration in mice subjected to a photoperiod of 13L:11D during the late afternoon inhibited the growth of fibrosarcomas. The growth of various carcinomas in different animals was enhanced after pinealectomy. They included ovarian carcinomas (Das Gupta 1968), Walker 256 (Rodin 1963), hormone-responsive hamster melanotic melanomas (Stanberry 1983), and mammary tumors (Blask 1984). Different carcinomas in different animals were found to be regressed after melatonin administration in the late afternoon. They included androgen-sensitive rat prostatic carcinomas, Lewis lung carcinomas (Lapin 1976), hormone-responsive hamster melanotic melanomas (Stanberry 1983), mouse melanoma cells (Narita & Kudo 1985), DMBA-induced mammary tumors (Blask 1986) and breast tumors (Karmali 1978). The growth of carcinomas in rats subjected to a short photoperiod was also suppressed. They included diethylstilboestrol-induced pituitary prolactinomas (Leadem 1987) and mammary tumors (Chang 1985).

Mice in some laboratories spontaneously developed mammary tumors which were known to have a viral etiology. Those mice which received melatonin in the drinking water were found to have a lower incidence of the mammary tumors than control mice which received alcohol in the drinking water. Subramanion (1991) suggested that melatonin was a suppressor of spontaneous murine mammary tumors.

1.7 Macrophages

Macrophages are mononuclear phagocytes which are able to ingest "non-self"

antigen-associated cells or substances and digest them in the lysosomal compartments. About 5 - 10 % macrophages carry class II major histocompatibility complex molecules and these macrophages are also antigen-presenting cells. They process antigens, associate the antigens with major histocompatibility complex molecules, and present the modified antigens to T lymphocytes and B lymphocytes. Macrophages produce and secrete IL-1 which is essential for T cell activation. α-Interferon, which is known as leukocyte interferon, is produced by macrophages. The secretory functions of macrophages are important for them to serve as effector cells. Neutral proteases including plasminogen activator, collagenase and elastase, and several potent oxidizing agents such as superoxide, nitrite and hydrogen peroxide are secreted by activated macrophages. Tumor necrosis factor - α is also secreted by activated macrophages (Old 1985). Macrophage activation is known to be divided into priming and activating stages. A low concentration of γ-interferon is required to initiate the priming stage. A high concentration of γ-interferon or lipopolysaccharide is then necessary for the primed macrophages to enter the activating stage.

Generally, macrophages are in either a free or a fixed state. Histiocytes, alveolar macrophages, peritoneal macrophages, and pleural macrophages are typical types of free macrophages. Fixed tissue macrophages of the spleen and lymph nodes, Kupffer cells of the liver, microglial cells of the nervous system, osteoclasts of the bone, and synovial type A cells of the joint are all typical types of fixed macrophages. All of the hematopoietic cells such as erythrocytes, granulocytes and mononuclear phagocytes are derived from the common pluripotent stem cells. Only after the appearance of the monoblasts, the first progenitor cells of macrophages, the

granulocytic series and the monocytic series are separated from the common committed stem cells. Monoblasts are round cells with few basophilic granules, Fc receptors and esterase reactivity. These monoblasts differentiate into promonocytes which stain azurophilic and possess myeloperoxidase-positive granules. The promonocytes mature into monocytes which have fewer myeloperoxidase-positive granules and a lower nucleus to cytoplasm ratio. These monocytes are released into the blood and circulate throughout the body. When the monocytes leave the circulating pool and emigrate into the extravascular environment, they further differentiate into mature macrophages under the influence of suitable mitogens such as macrophage specific colony stimulating factor.

Migration of macrophages may be in a random manner or specifically direct toward a chemical stimulus, i.e. chemotaxis. When macrophages are attracted toward a specific location, they must be prevented from random migration by some factors. Substances responsible for retention of macrophages are known to be lymphokines such as macrophage migration inhibitory factor and macrophage activation factor, proteolytic enzymes such as factor Bb of the complement activation system, and plasmin of the fibrinolytic system. Substances which directed the movement of macrophages are named chemotactic agents. C5a, which is an anaphylatoxin produced in the activation of complement by antigen-antibody complexes in both classical and alternative pathways, is also a typical chemotactic agent. Bacterial products like N-formylmethionyl peptides and some products from stimulated T and B lymphocytes also show chemotactic activities.

Several lines of evidences suggest the involvement of macrophages in the growth of tumors. Macrophages often infiltrate into tumors. Macrophages explanted from regressing tumors are more cytotoxic than macrophages explanted from progressing tumors. The size of the macrophage population infiltrating into the tumors seems to correlate with the metastatic ability of the tumor. Immunotherapy against tumors always elicits infiltration of mononuclear phagocytes. Injection of bacille Calmette-Guerin or Corynebacterium Parvum for immunostimulation enhances the ability of the recipient's macrophages to kill tumors in vitro (Nathan 1980). Melatonin has shown its antitumor potential both in vitro and in vivo. The effects of melatonin on macrophages were hence studied in this project.

1.8 Lymphocytes

Lymphocytes are divided into T lymphocytes and B lymphocytes. T lymphocytes are derived from the thymus. When T cells mature, they migrate from the cortex to the medulla of the thymus. Matured T cells move into efferent lymphatics, large lymphatic ducts, then into the thoracic ducts, and finally into the bloodstream. They are localized in different organs such as lymph nodes, spleen, Peyer's patches, etc. When the stem cells move into the thymus, they begin to express the CD2 molecules, which are receptors by which T cells form rosettes with the sheep red blood cells. Then they further differentiate into $CD2^+CD3^+CD4^+$ T cells or $CD2^+CD3^+CD8^+$ T cells. $CD4^+$ T cells comprise 65 % peripheral T cells while $CD8^+$ T cells comprise 35 % peripheral T cells. The CD4 molecules on T cells interact with antigens associated with class II major histocompatibility complex molecules. The CD8

molecules on T cells interact with antigens associated with class I major histocompatibility complex molecules. The activation of T cells requires several signals (Roitt 1989). First, IL-1 secreted by the activated macrophages, antigens associated with suitable major histocompatibility complex molecules, or mitogens activate 2 signals in the T cells. Second, an increase in inositol triphosphate elevates the cytoplasmic level of free calcium by mobilizing calcium from the intracellular stores in the endoplasmic reticulum. Third, an increase in diacylglycerol activates protein kinase C. These signals act in concert to initiate growth and proliferation of T cells. Further developments of T cells require IL-2, which is secreted by activated T cells and known as T cell growth factor, and the number of IL-2 receptors on the activated T cells increased. IL-2 is also necessary for the development of cytotoxic effector $CD8^+$ T cells. Activated T cells secrete migration inhibiting factor which inhibits the random migration of activated macrophages and makes them accumulate around the area of activation. Activated T cells also secrete γ-interferon which enhances the cytolytic activity of the accumulated macrophages.

B cells are derived from stem cells which are first located in the fetal liver and subsequently migrate to the bone marrow. The stem cells differentiate into pre-B cells. They lack immunoglobulins at first and are able to synthesize the heavy chains of immunoglobulins later. These lymphoblasts are unable to produce the light chains of immunoglobulins. The pre-B cells enter into the resting stage of B cells when they express surface immunoglobulins. Several surface markers are carried by the different stages of B cells. The resting B cells are δ^+/Ba^-, IL-4 R^+, IL-6 R^- (R denotes the receptor, + indicates presence, - indicates absence). The previous process

does not require an antigen. Activation of resting B cells into activated and mature stages requires T-dependent antigens or T-independent antigens. The B cells ingest the antigens either specifically or nonspecifically, internalize them and express the antigens with class II major histocompatibility complex molecules which are then recognized by the T cell antigen receptor on T_H cells. IL-4 which is secreted by T_H cells and known as B cell growth factor and the IL-1 secreted by the macrophages stimulate the resting B cells to enlarge and enter the cell cycle. The activated B cells then change into δ^-/Ba^+, IL-4 R^{++}, and IL-6 R^+. IL-2, IL-4, IL-5 and Υ-interferon are required for the proliferation of the activated B cells. IL-6, which is the most important differentiation factor of B cells, IL-2, IL-4, IL-5, and Υ-interferon are then required for the differentiation of B cells. The mature B cells finally change into δ^-/Ba^{++}, IL-4 R^+, and IL-6 R^{+++}. There is a down-regulation of IL-4 receptors and an up-regulation of IL-6 receptors. The B cells may change into active antibody-producing plasma cells. They may change into memory cells to wait for the initiation of secondary immune response by the same antigen. The memory cells are δ^-/Ba^-. In addition to the help of the lymphokines from T cells, B cells can be activated by T-independent antigens through either receptor cross-linking or mitogenic action. The receptor cross-linking leads to activation of phospholipase C and generation of 2 second messengers within seconds. It results in an increase of cytoplasmic Ca^{++} and activation of protein kinase C. Mitogens are believed to lead to an increase in the level of calcium ions. They also act in concert to lead to an activation of B lymphocytes.

Chapter 2 Toxicity of pineal indoles on tumor cell lines

2.1 Introduction

The relationship between melatonin and cancer has been reviewed by Blask and Hill (1988). The general consensus is that the pineal is an oncostatic gland (Kerenyi et al., 1990) although there was some evidence to the contrary.

Melatonin inhibited estradiol-induced ^{3}H-thymidine incorporation into the DNA of MCF - 7 human breast cancer cells (Blask and Hill, 1986a). It also suppressed the growth of fibrosarcomas (Bartsch and Bartsch, 1981), prostatic carcinomas (Philo and Berkowitz, 1988; Toma et al., 1988), melanomas (Narita and Kudo, 1985), 7,12-dimethylbenzanthracene-induced mammary tumors (Blask et al., 1986) and spontaneous mammary tumors (Subramanian and Kothari, 1991). Bartsch and Bartsch (1981) observed in mice that melatonin administration suppressed the growth of Erlich's solid tumors. Lapin and Frowein (1981) noted in rats that pinealectomy promoted the growth of Yoshida solid tumors and that melatonin reversed the effect of pinealectomy and prolonged survival time. Pinealectomy enhanced growth of ovarian carcinomas (Das Gupta, 1968) and Walker 256 carcinomas (Barone and Das Gupta, 1970) and accelerated metastasis of the latter (Barone and Das Gupta, 1970). However, in human cancer patients no consistent changes in the secretory pattern of melatonin compared with healthy individuals could be detected (Blask and Hill, 1988).

In addition to melatonin, other pineal products have been found to possess antitumor activity. A low molecular weight and protease-resistant compound, distinct from melatonin, serotonin, pteridines and ß-carboline, with an inhibitory effect on the growth of human melanoma cells in vitro, was purified from an aqueous extract of ovine pineals (Noteborn et al., 1988). Shellard et al. (1989) showed that methoxytryptamine was more potent than melatonin in inhibiting growth of several tumor cell lines including SK-OV-3, JA-1 (ovarian carcinoma cells), RT112 (bladder tumor cells) and MCF-7 (breast tumor cells).

The intent of the present investigation was to examine the effect of melatonin together with other pineal indoles on a variety of tumor cell lines including fibroblasts (3T3), macrophage-like cell line (PU5), mouse melanoma (B16) and choriocarcinoma (JAr).

2.2 Material and methods

2.2.1 *Cell lines*

The mouse melanoma cell line (B16) was obtained from Dr. K. Yamada, Department of Dermatology, University of Tokyo. The human choriocarcinoma cell line (JAr) was obtained from Dr. R.A. Patillo, Medical College of Wisconsin. The sarcoma cell line (S180), macrophage-like cell line (PU5) and fibroblast cell line (3T3) were obtained from American Type Culture Collection. The PU5 and 3T3 cell lines were routinely maintained as monolayers in RPMI 1640 (Sigma, catalog no. R 6504). The

B16 cell line was routinely maintained as monolayers in Nutrient Mixture F-10 Ham (Sigma, catalog no. R 6635). The S180 and JAr cells were maintained as suspension cells in RPMI 1640. All culture media were supplemented with 10% fetal calf serum and streptomycin sulphate (100 μg/ml) and penicillin G (100 IU/ml). Dulbecco's phosphate buffered saline (Sigma, catalog no. D 5652) was used for washing. Generally, they were first unpacked and dissolved in 800 ml fresh double-distilled water. They were stirred gently for 3 - 4 hours. 2 g sodium bicarbonate was then added to RPMI, 1.2 g sodium bicarbonate was added to F10 and 0.1 g $MgCl_2 \cdot 6\ H_2O$ and 0.133 g $CaCl_2 \cdot 2\ H_2O$ were added to Dulbecco's phosphate-buffered saline. The solution was stirred for a few minutes. The pH of the solution was adjusted to 7.2 while stirring. Then the volume of the solution was adjusted to 1 L with fresh double-distilled water. Finally, the medium was sterilized by filtration using sterile 0.22 μm millipore filter. The sterile medium was then transferred to a sterile container for storage.

2.2.2 *Incorporation of radioactive precursors*

Radioactive precursors (methyl-^{3}H-thymidine, L-4,5-^{3}H-leucine, 5-^{3}H-uridine) were purchased from Amersham. Cells in the exponential growth phase were used. Cells (10^4) in triplicate were seeded into each well of 96-well plates and incubated for 48 hours with different concentrations of serotonin, melatonin, hydroxyindoleacetic acid, methoxytryptophol, methoxytryptamine, methoxyindoleacetic acid and hydroxytryptophol (Sigma Chemical Co.). Radioactivity (0.5 μCi/10 μl) was added to each culture well at the end of experiment and incubation continued for another

6 hours. The cultures were then harvested by a cell harvester. The incorporated radioactivity was determined by liquid scintillation counting.

2.2.3 *Preparation of scintillation fluid*

0.4 g POPOP and 4 g PPO were dissolved in 700 ml toluene and the mixture was stirred for 3 hours. 300 ml Triton X-100 was then added into the mixture to bring the volume to 1 L.

2.2.4 *Determination of cell viability by uptake of crystal violet*

After incubation with pineal indoles for 48 hours the cells were subjected to staining with crystal violet (0.5 % in methanol/water : 1/4 by volume) for 3 minutes. Afterwards they were washed with phosphate buffered saline twice and then dried. They were lysed by lysis buffer which contains 1 % acetic acid - 50 % alcohol. The quantity of crystal violet taken up by the cells was determined using a micro ELISA autoreader (3550 Bio-Rad microplate reader) at 595 nm.

2.2.5 *Hepatocyte experiment*

Rat hepatocytes were isolated from male Lewis rats according to the method described by Kreamer et al. (1986). Rats weighing 250 g were anesthetized with sodium pentabarbital and the livers were first perfused with warm Ca^{++}-free Hank's balanced salt solution (HBSS) fortified with 15 mM HEPES to remove blood cells

before the liver tissues were perfused with 100 ml HBSS containing 50 mg collagenase (Type IV from Clostridium Histolyticum, Catalog no. C5138, Sigma, USA) for 10 minutes. The perfusion rate and temperature were maintained at 20 ml/min and 35 °C. The digested liver was then removed and dissociated mechanically in 50 ml of collagenase - supplemented perfusate. Liver cells were obtained by filtering the solution through a sterile nylon mesh (250 μm pore size, Baltex 25-T, Polyester Monofilament, Switzerland) and then centrifuging at 50 g for 3 min. The cells were rinsed twice in Medium M199 supplemented with glucose (1.5 g/l), streptomycin (100 mg/l), penicillin (100 IU/ml), insulin (0.5 mg/l), transferrin (5 μg/ml), sodium selenite (5 ng/ml) and 10% fetal calf serum, counted and resuspended to a concentration of 5 to 10 x 10^6 cells/ml fresh medium. Twenty-five milliliters of liver cell suspension was mixed with 24 ml of sterile isosmotic percoll solution to produce a final density of 1.06 g/ml. Cells with about 90 to 95 % viability were obtained by centrifuging the suspension for 10 min at 50 g. The cells were rinsed twice, counted and cultured in 96-well culture plates (1 x 10^4 cells/0.1 ml/well) for 3 h. The cells were rinsed briefly with warmed medium and cultured in the presence of pineal indoles. The activities of glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase in the culture medium were determined as described by Reitman (1957). Cell viability was estimated by uptake of crystal violet.

2.2.6 *Statistics*

The Data was analyzed by one way analysis of variance using Scheffe's multiple

range test. The levels of significance were taken at $p < 0.05$.

2.3 Results

Methoxytryptamine inhibited ^{3}H-thymidine incorporation by mouse melanoma (B16) cells with the highest potency (Figure 2.1). Marked inhibition of ^{3}H-uridine incorporation (Figure 2.2) and ^{3}H-leucine incorporation (Figure 2.3) by mouse melanoma cells was also achieved by methoxytryptamine. The effects of the other indoles were much weaker.

Methoxytryptamine exhibited the highest potency of inhibiting ^{3}H-thymidine incorporation by sarcoma (S180) cells (Figure 2.4). The activities of most of the other indoles were weak up to 100 μM. Serotonin and hydroxyindoleacetic acid had low potencies even at 500 μM.

Little inhibition (< 20 %) of ^{3}H-thymidine incorporation by PU5 cells in the presence of the various pineal indoles was observed up to an indole concentration of 100 μM (Figure 2.5). At 500 μM concentration only methoxytryptamine demonstrated over 90 % inhibition.

At the concentration of 4 and 20 μM hydroxytryptophol appeared to be the most potent inhibitory indole in the inhibition of ^{3}H-thymidine incorporation by choriocarcinoma (Figure 2.6). At the concentration of 100 and 500 μM serotonin appeared to be the least potent. All of the tested pineal indoles except serotonin and

hydroxyindoleacetic acid achieved over 90 % inhibition at the concentration of 500 μM.

Methoxytryptamine exerted the most potent inhibitory effect (approximately 40 % inhibition at 100 μM and 500 μM) on crystal violet uptake by the fibroblast (3T3) cell line. The degree of inhibition achieved by the other pineal indoles was in general below 20% (Figure 2.7).

The ID_{50}s (dosages required to achieve 50 % inhibition of cell growth) of pineal indoles with regard to various cell lines were shown in Table 1. The order of potency was methoxytryptamine >> methoxytryptophol and hydroxytryptophol > melatonin and methoxyindoleacetic acid > serotonin and hydroxyindoleacetic acid.

When melatonin and methoxytryptamine were tested for possible toxic effects on rat hepatocytes in primary culture, it was found that the pineal indoles did not increase the release of glutamate-oxaloacetate transaminase by hepatocytes into the culture medium (Figure 2.8). However, the release of glutamate-pyruvate transaminase was slightly enhanced (Figure 2.9). There was a slight inhibition of the uptake of crystal violet by both indoles (Figure 2.10).

2.4 Discussion

Melatonin was found to have a low potency in suppressing the growth of the tumor cell lines. It is consistent with the findings of Boucek and Alvarez (Boucek 1970)

and Shellard (1989). Methoxytryptamine exerted the highest potency in suppression of the growth of tumor cell lines. The demonstration of the suppressive effects of pineal indoles on in vitro ^{3}H-thymidine incorporation by sarcoma and mouse melanoma cells in the present study is consistent with the findings reported by Bartsch and Bartsch (1981) and Narita and Kudo (1985) that melatonin slowed down the growth of these tumor cell lines in vivo. The inhibitory effect of the pineal indoles on ^{3}H-thymidine incorporation by choriocarcinoma cells is reminiscent of the action of melatonin on neoplasms in the female reproductive tract like ovarian carcinoma (Shellard et al., 1989) and endometrial cancer (Blask and Hill, 1986b). Hill (1986) observed that at low concentrations (1-100 nM), melatonin, N-acetylserotonin and serotonin did not affect proliferation of the murine 3T3 cell line. Results of the present investigation are in agreement with his data. Methoxytryptamine was the most potent inhibitory indole because it was still effective at the low concentration of 10 μM on mouse melanoma and sarcoma cells.

The present study revealed that methoxytryptamine was more potent than melatonin or any other pineal indoles in inhibiting the incorporation of ^{3}H-thymidine in most tumor cell lines.

In previous studies methoxytryptamine has been shown to be more active than other pineal indoles in inhibiting LH-stimulated testosterone production by isolated rat Leydig cells (Ng and Lo, 1988), ACTH-stimulated corticosterone and aldosterone production by isolated rat adrenal cells (Ng, 1987), epinephrine-stimulated lipolysis in isolated rat adipocytes (Ng and Wong, 1986) and spermatogenesis in hamsters (Ooi

and Ng, 1989). Thus it is interesting to note that methoxytryptamine is the pineal indole with the highest cytotoxic effect on tumor cell lines.

The demonstrated effect of pineal indoles on the tumor cell lines represents a direct cytotoxic action rather than an indirect one through their immunomodulatory action (Maestroni et al., 1986, 1987a, 1987b, 1988). In the in vivo condition the antitumor action of melatonin (Blask and Hill, 1988) may be expressed through a direct cytotoxic effect and also through an indirect immunomodulatory effect. In the present study reduction of incorporation of ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine of tumor cell line after incubation with pineal indole may indicate inhibition of synthesis of DNA, RNA and protein.

It is noteworthy that the pineal indoles did not exert significant toxic effects on isolated rat hepatocytes as reflected by results of the liver enzyme assays and dye uptake assays.

Results of the present investigation are in general in support of evidence in the literature on the antitumor/oncostatic effect of pineal/melatonin. It has disclosed that melatonin is not the only antitumor indole elaborated by the pineal. Blask and Hill (1988) hold the opinion that melatonin constitutes a potentially useful substance for cancer chemotherapy. The other pineal indoles, perhaps methoxytryptamine, might one day turn out to be useful also in the same regard.

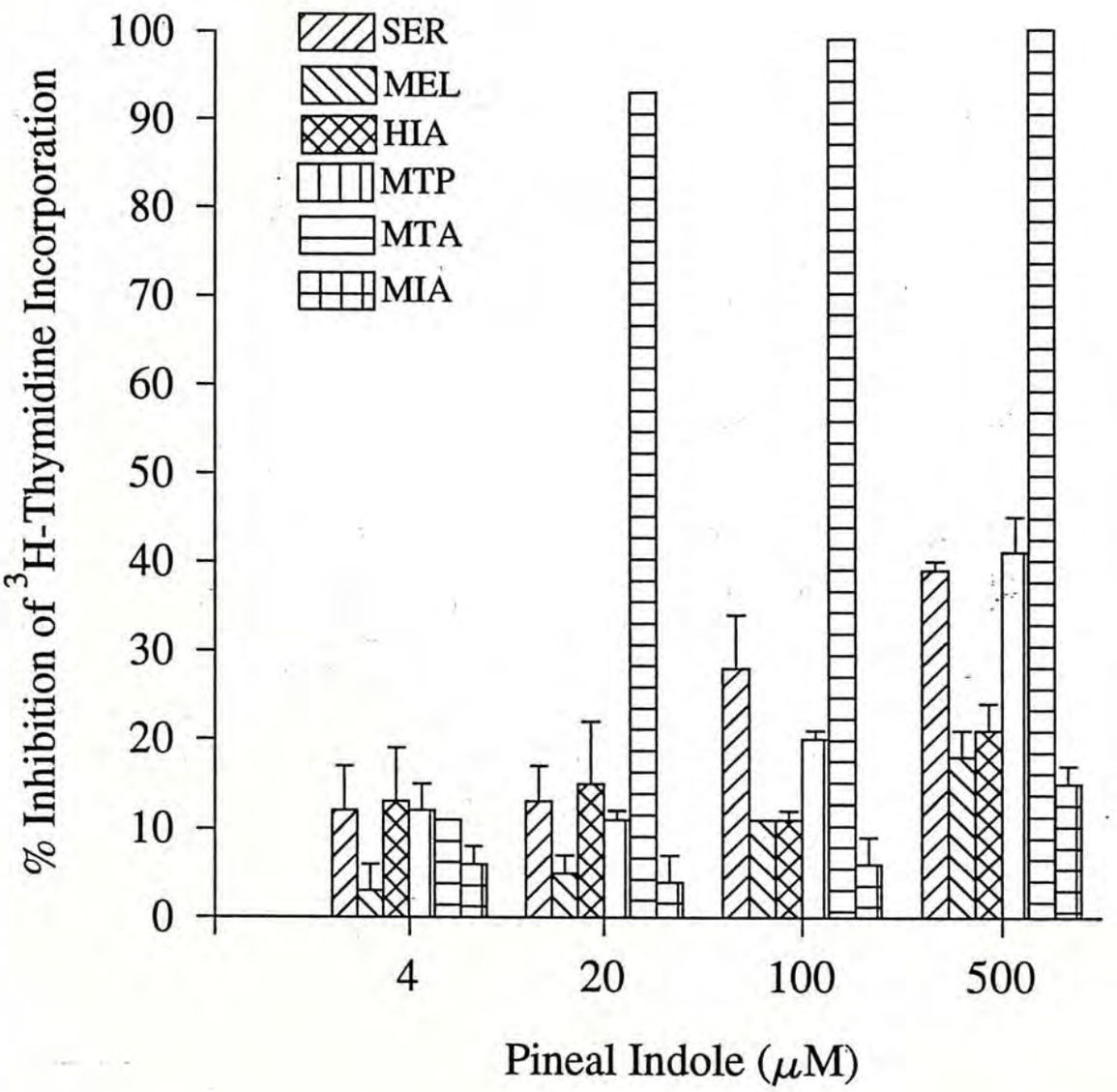


Figure 2.1 Effects of pineal indoles on ^{3}H-thymidine incorporation by mouse melanoma (B16) cells after 48 hours of incubation. Results are expressed as means ± standard deviation (n = 3). SER = serotonin, MEL = melatonin, HIA = hydroxyindoleacetic acid, MTP = methoxy - tryptophol, MTA = methoxytryptamine, MIA = methoxyindoleacetic acid.

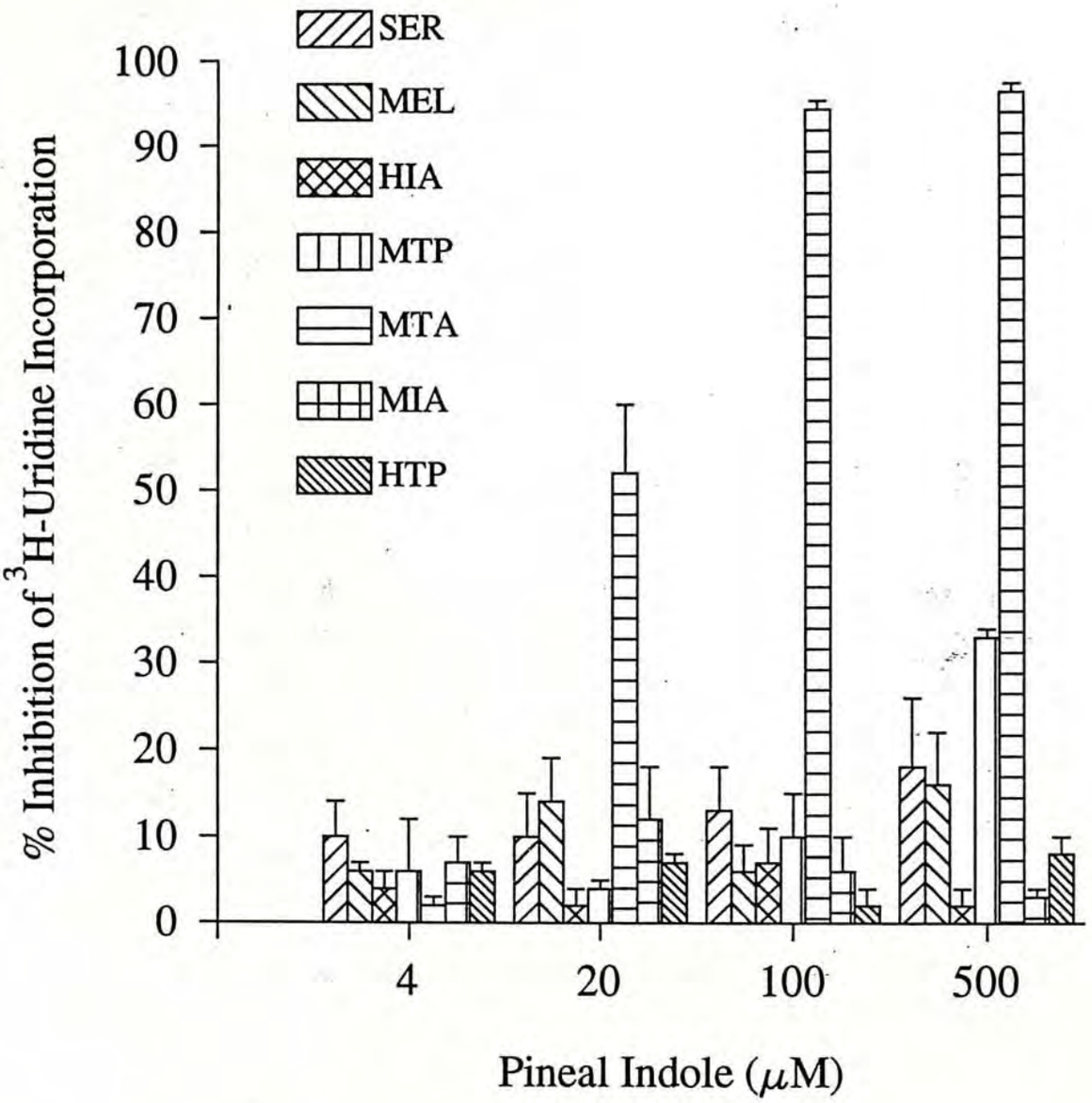


Figure 2.2 Effects of pineal indoles on ^{3}H-uridine incorporation by mouse melanoma (B16) cells after 48 hours of incubation. Results are expressed as means ± standard deviation (n = 3). SER = serotonin, MEL = melatonin, HIA = hydroxyindoleacetic acid, MTP = methoxy - tryptophol, MTA = methoxytryptamine, MIA = methoxyindoleacetic acid, HTP = hydroxytryptophol.

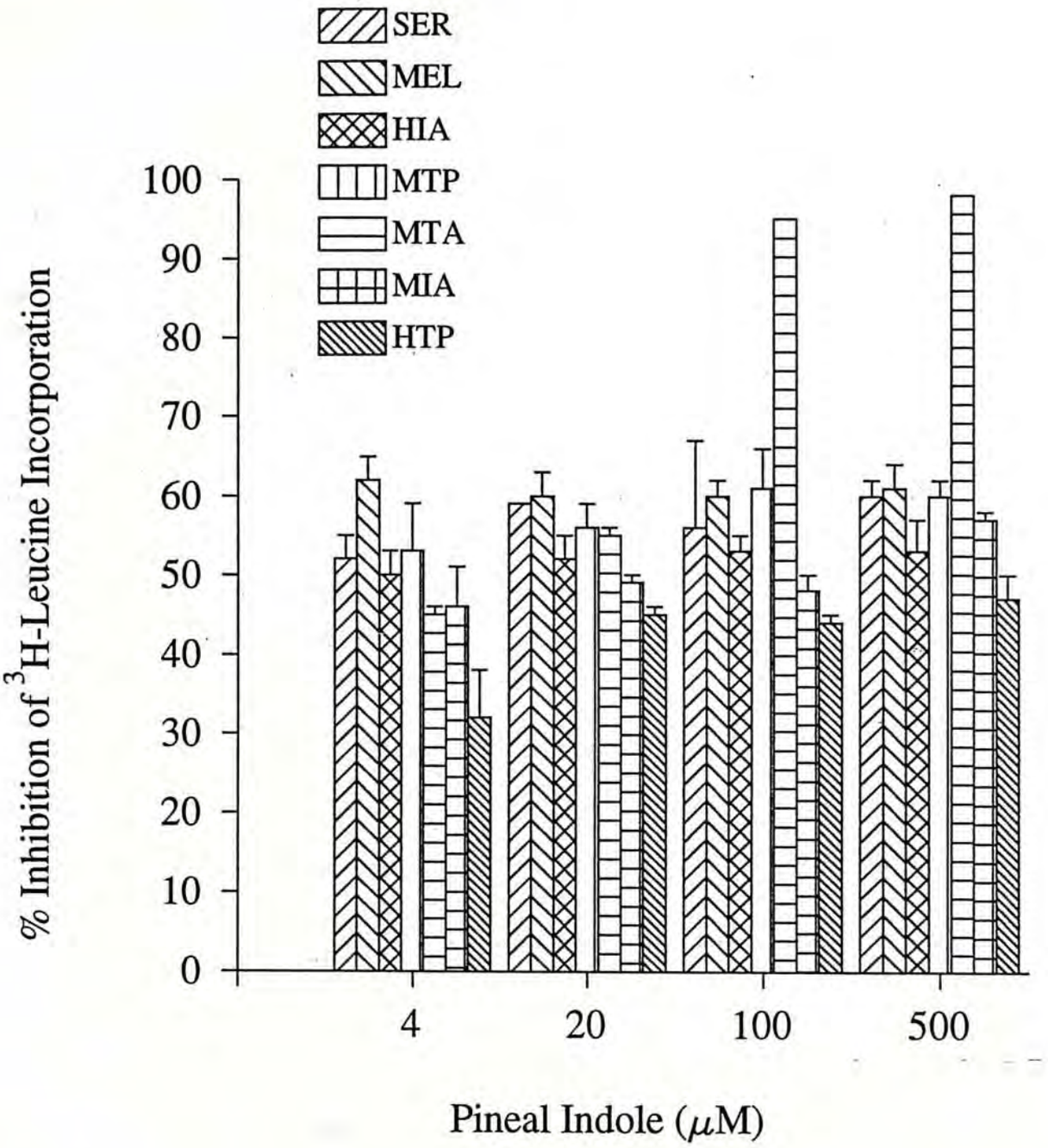


Figure 2.3 Effects of pineal indoles on ^{3}H-leucine incorporation by mouse melanoma (B16) cells after 48 hours of incubation. Results are expressed as means ± standard deviation (n = 3). SER = serotonin, MEL = melatonin, HIA = hydroxyindoleacetic acid, MTP = methoxy - tryptophol, MTA = methoxytryptamine, MIA = methoxyindoleacetic acid, HTP = hydroxytryptophol.

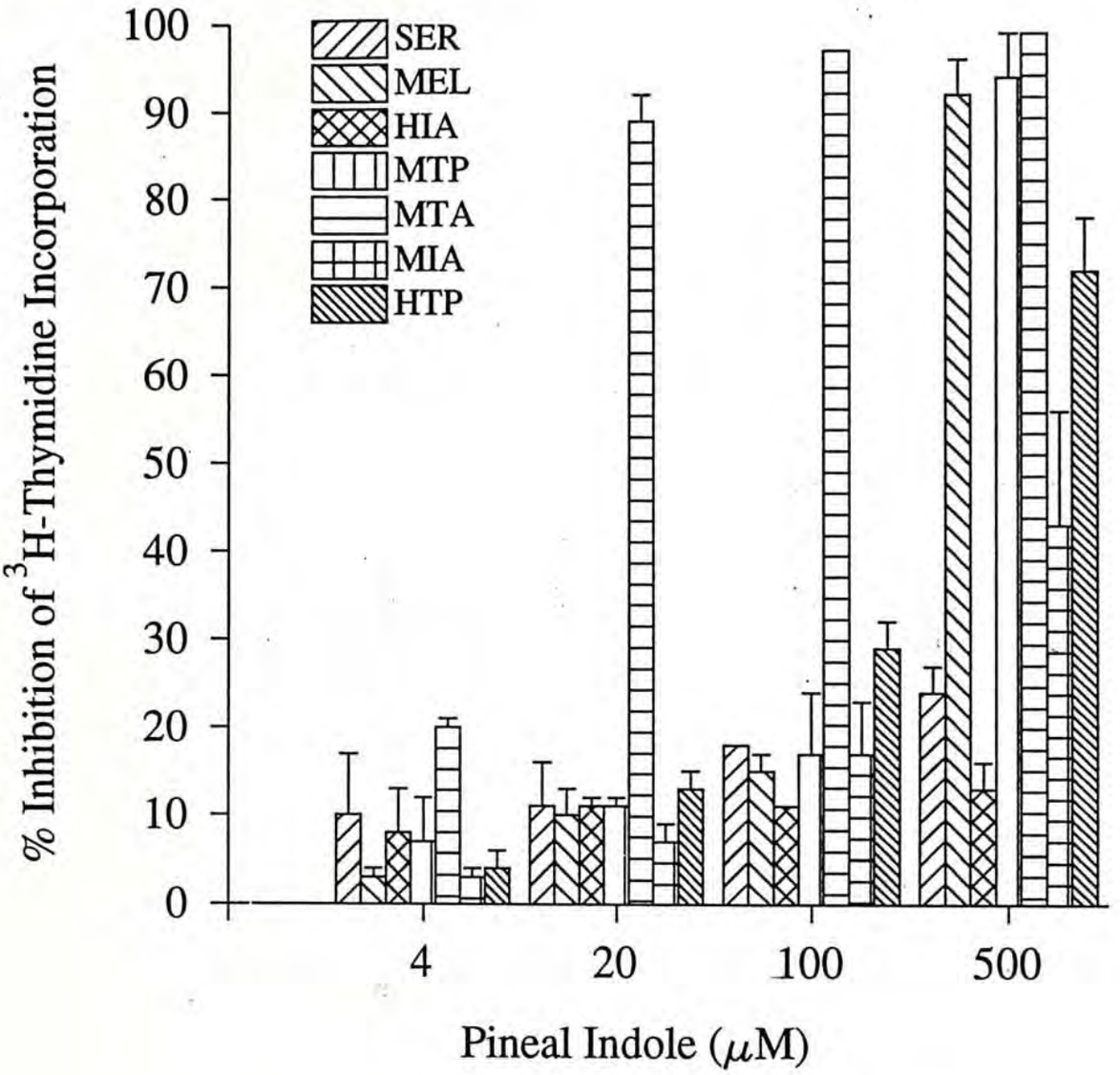


Figure 2.4 Effects of pineal indoles on ^{3}H-thymidine incorporation by sarcoma (S180) cells after 48 hours of incubation. Results are expressed as means ± standard deviation (n = 3). SER = serotonin, MEL = melatonin, HIA = hydroxyindoleacetic acid, MTP = methoxytryptophol, MTA = methoxytryptamine, MIA = methoxyindole-acetic acid, HTP = hydroxytryptophol.

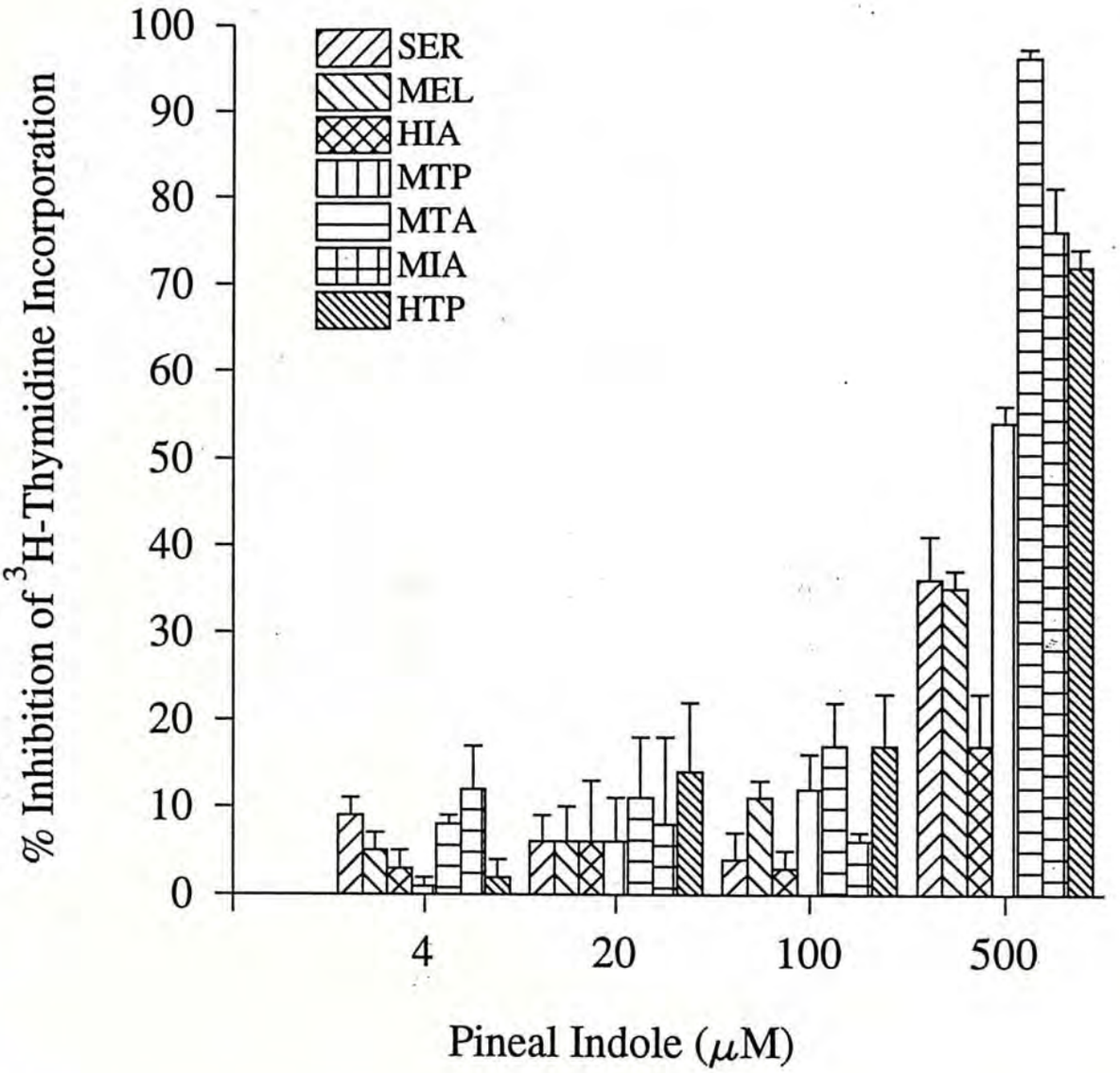


Figure 2.5 Effects of pineal indoles on ^{3}H-thymidine incorporation by macrophage-like (PU5) cells after 48 hours of incubation. Results are expressed as means ± standard deviation (n = 3). SER = serotonin, MEL = melatonin, HIA = hydroxyindoleacetic acid, MTP = methoxy - tryptophol, MTA = methoxytryptamine, MIA = methoxyindoleacetic acid, HTP = hydroxytryptophol.

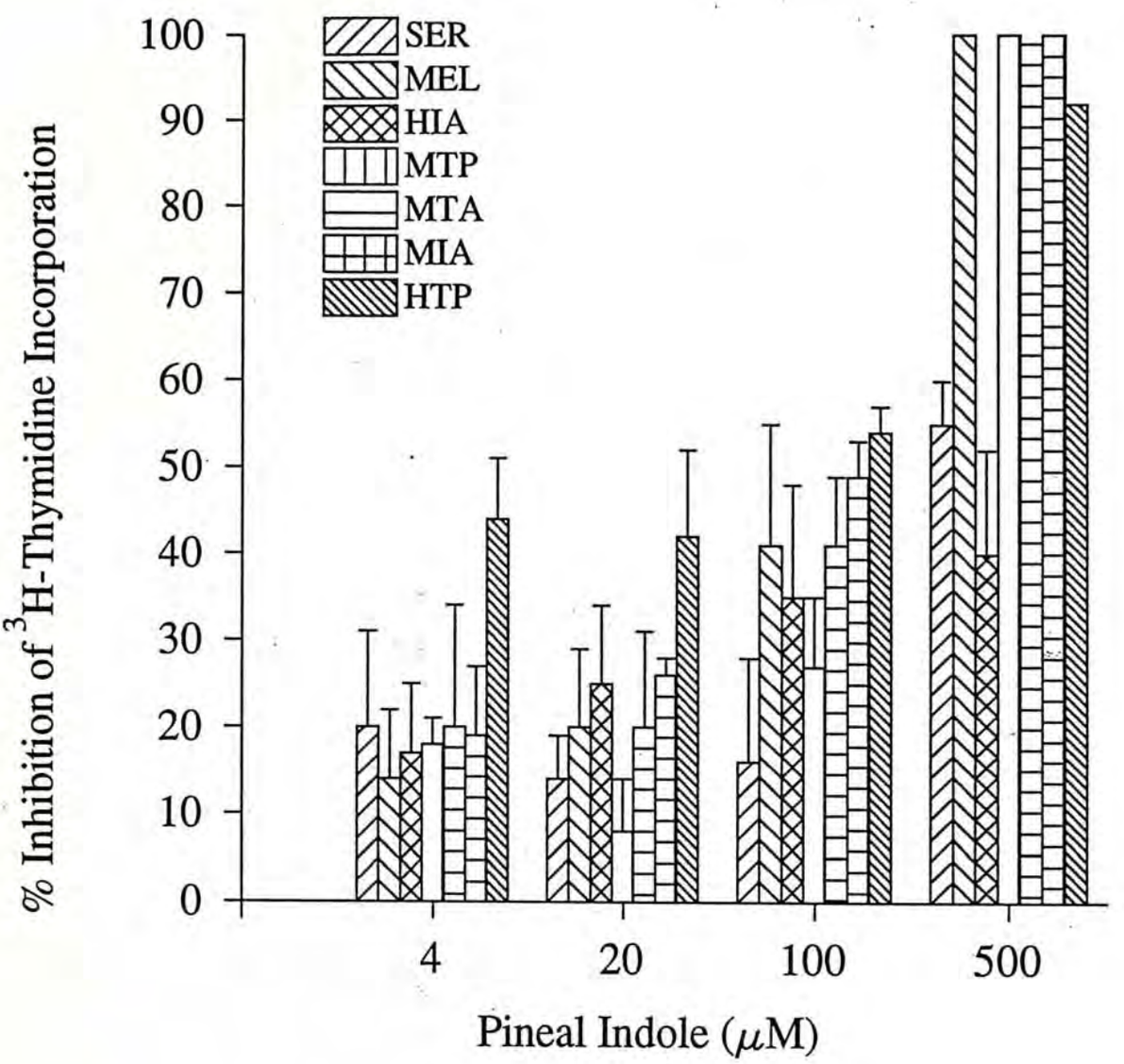


Figure 2.6 Effects of pineal indoles on ^{3}H-thymidine incorporation by choriocarcinoma (JAr) cells after 48 hours of incubation. Results are expressed as means ± standard deviation (n = 3). SER = serotonin, MEL = melatonin, HIA = hydroxyindoleacetic acid, MTP = methoxy - tryptophol, MTA = methoxytryptamine, MIA = methoxyindoleacetic acid, HTP = hydroxytryptophol

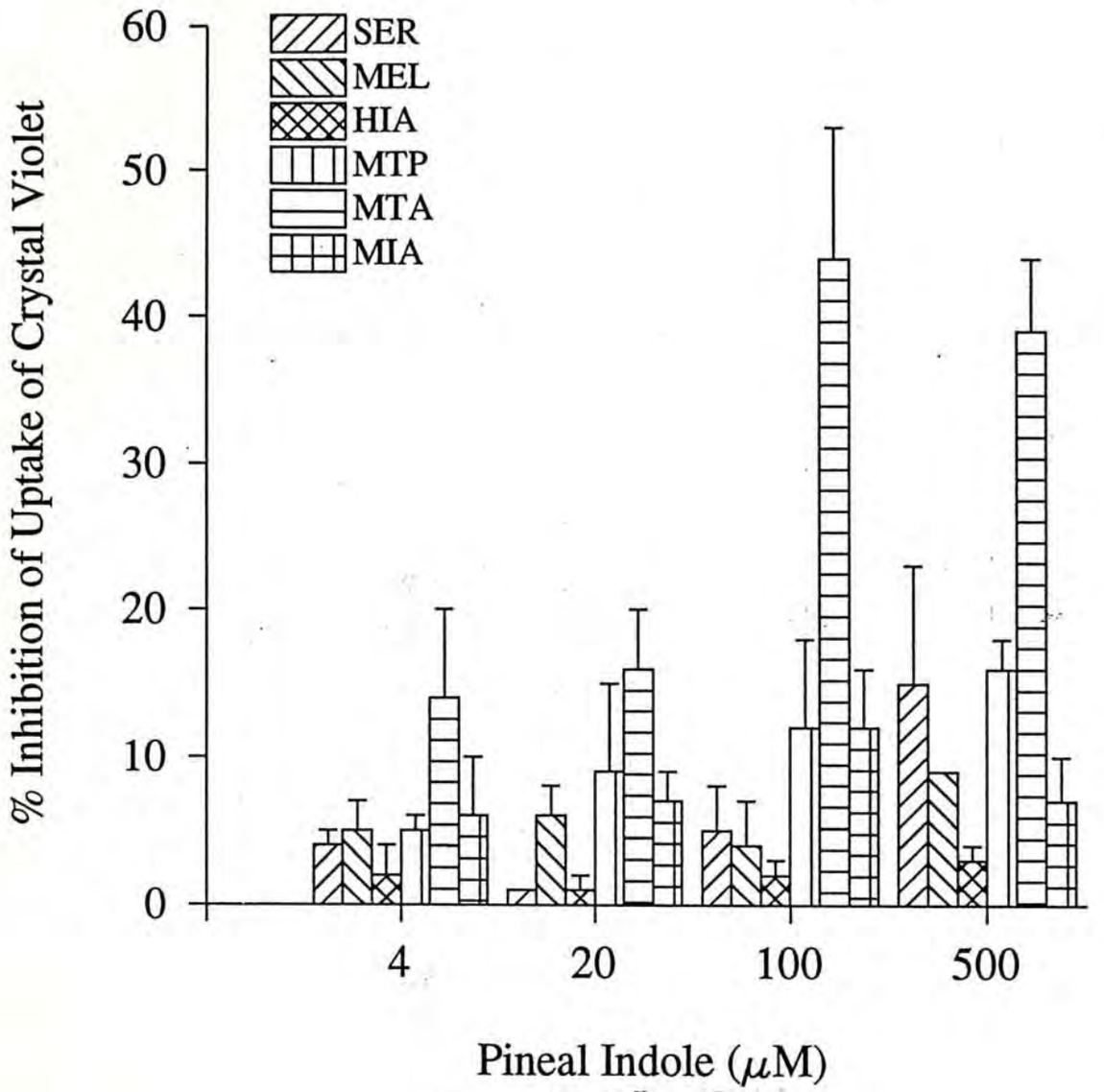


Figure 2.7 Effects of pineal indoles on uptake of crystal violet by fibroblasts (3T3) after 48 hours of incubation. Results are expressed as means ± standard deviation (n = 3). SER = serotonin, MEL = melatonin, HIA = hydroxy - indoleacetic acid, MTP = methoxytryptophol, MTA = methoxytryptamine, MIA = methoxyindoleacetic acid.

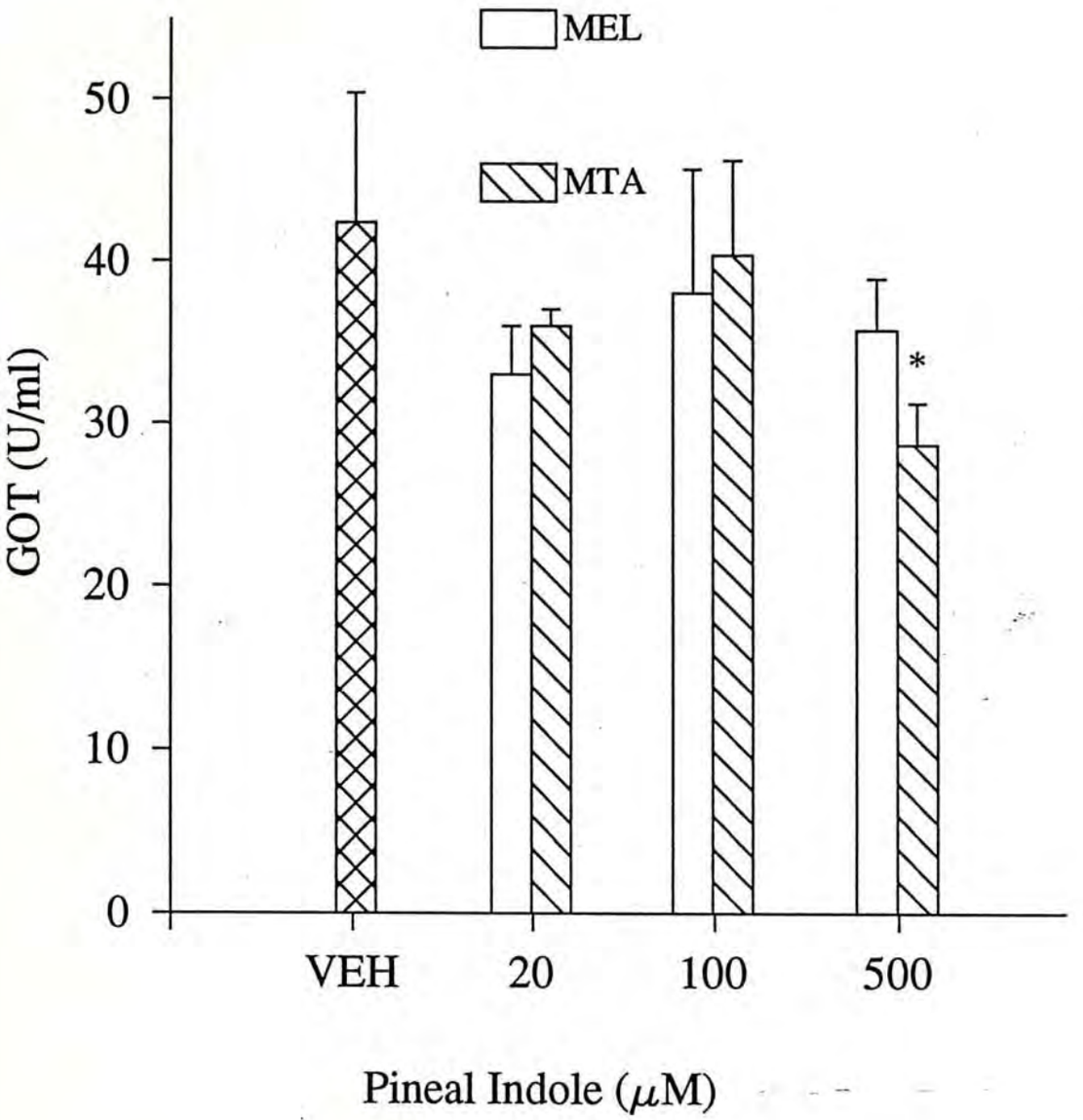


Figure 2.8 In vitro effects of melatonin and methoxytryptamine on release of glutamate oxaloacetate transaminase by a primary culture of rat hepatocytes. Results are expressed as means ± standard deviation (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

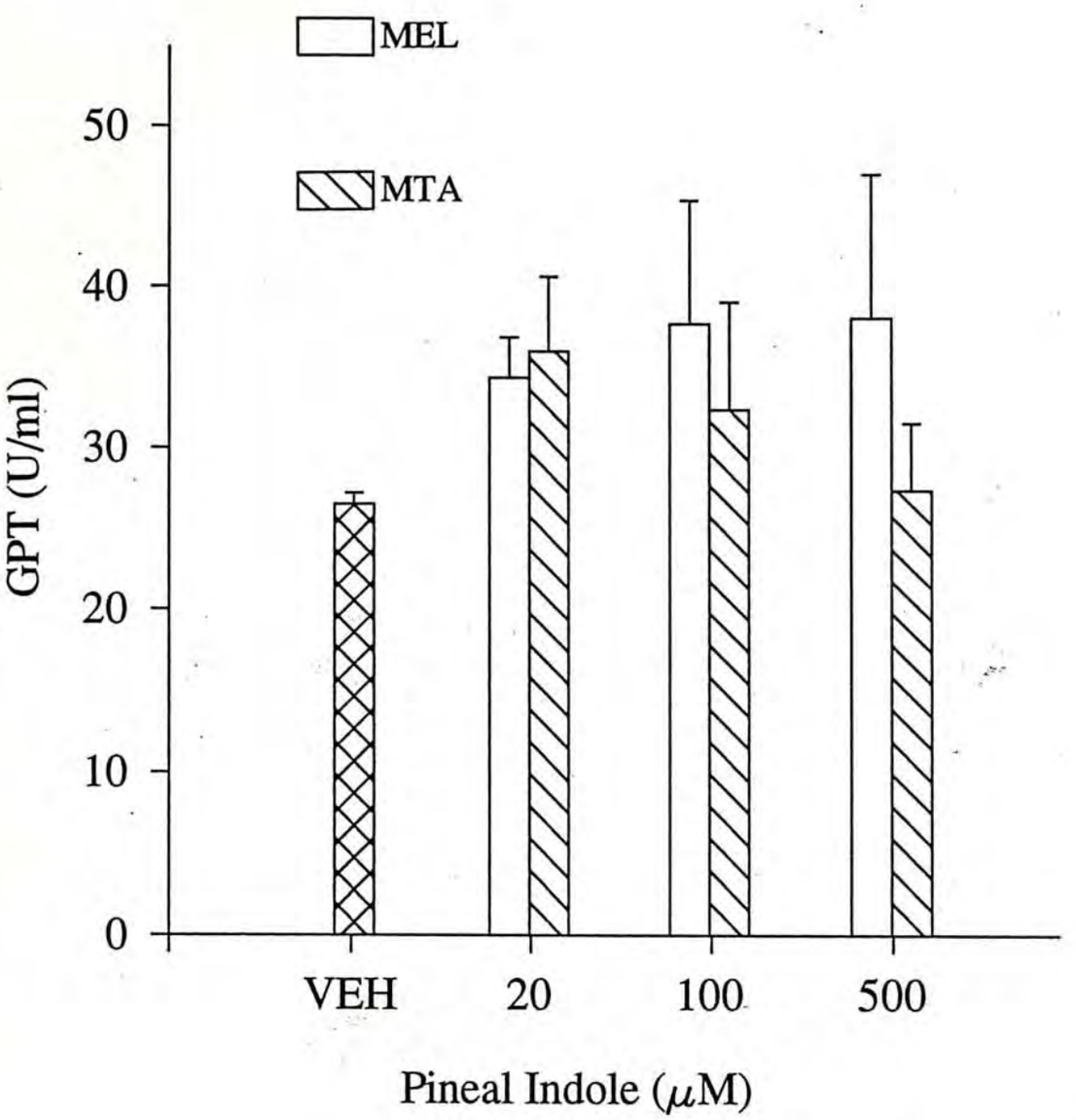


Figure 2.9 In vitro effects of melatonin and methoxytryptamine on release of glutamate pyruvate transaminase by a primary culture of rat hepatocytes. Results are expressed as means ± standard deviation (n = 3). VEH = control, MEL = melatonin, MTA = methoxy-tryptamine.

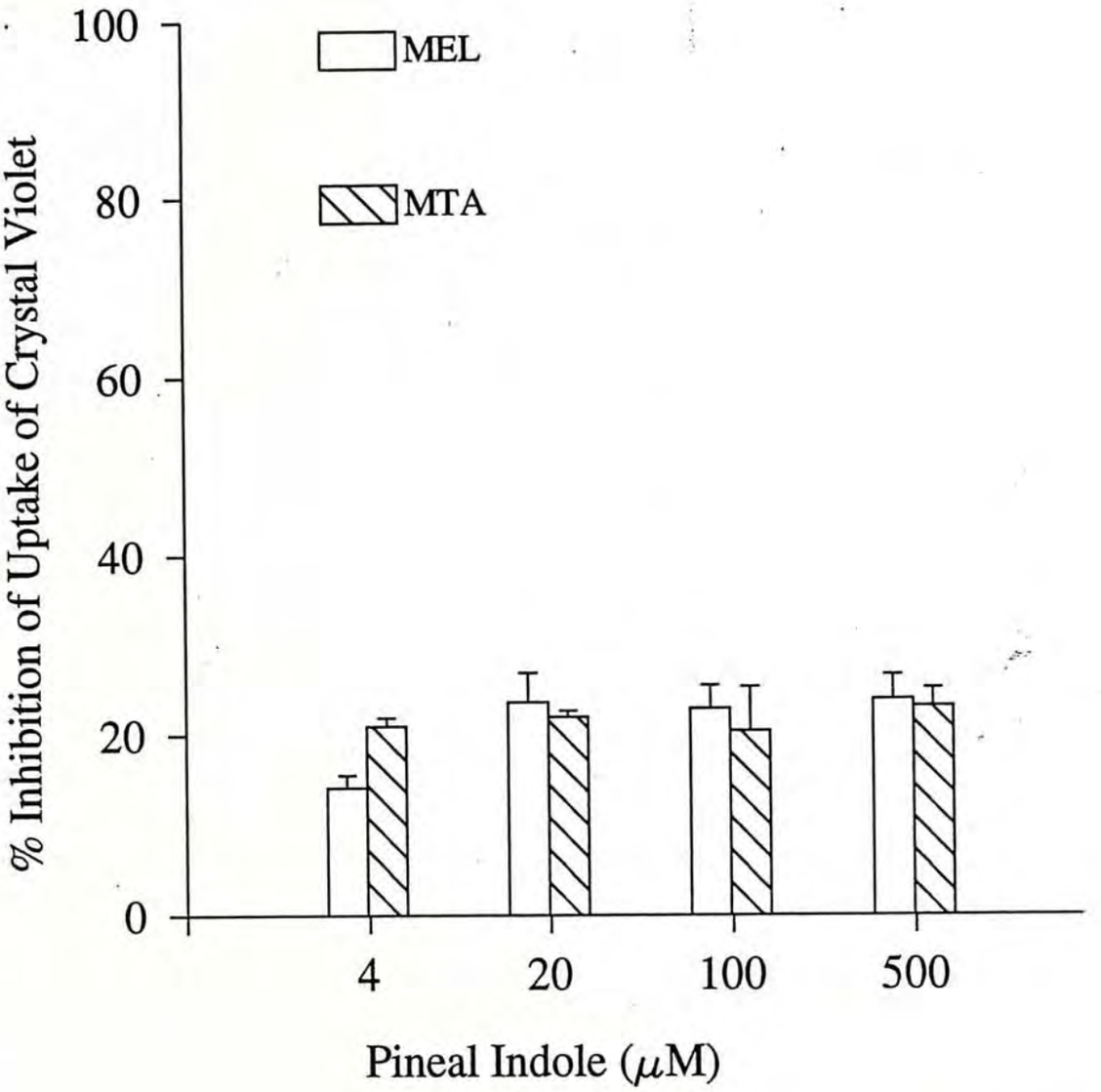


Figure 2.10 In vitro effects of melatonin and methoxytryptamine on uptake of crystal violet by a primary culture of rat hepatocytes. Results are expressed as means ± standard deviation (n = 3). VEH = control, MEL = melatonin, MTA = methoxytryptamine.

Table 1 Doses of pineal indoles (μM) required to achieve 50 % inhibition of ^{3}H-thymidine uptake by various cell types.

	SER	MEL	HIA	MTP	MTA	MIA	HTP
3T3	>500	>500	>500	>500	100	>500	------
B16	>500	>500	>500	>500	11	>500	------
PU5	>500	>500	>500	462	267	345	340
S180	>500	282	>500	271	11	>500	295
JAr	448	161	500	226	162	108	50

SER = Serotonin, MEL = melatonin, HIA = hydroxyindoleacetic acid, MTP = methoxytryptophol, MTA = methoxytryptamine, MIA = methoxyindoleacetic acid, HTP = hydroxytryptophol

Chapter 3 Activation of murine peritoneal macrophages by melatonin and methoxytryptamine

3.1 Introduction

The suppressive effect of melatonin and methoxytryptamine on the growth of tumors has been demonstrated in the previous chapter. Earlier melatonin was shown to inhibit the growth of tumors including breast tumors (Karmali 1978), fibrosarcomas (Bartsch 1981), Lewis lung carcinomas (Lapin 1976), melanomas (Narita 1985, Stanberry 1983), mammary tumors (Blask 1986), and prostatic carcinomas (Philo 1988, Toma 1987). Since involvement of macrophages in tumor growth is well known, the possibility of an immunomodulatory effect of melatonin and its derivatives on macrophages was hence studied.

Tumoricidal activities of macrophages can be generally divided into antibody - dependent cellular cytotoxicity (ADCC) and antibody - independent macrophage - mediated tumor cytotoxicity (Fan 1991). This study focused mainly on the antibody - independent macrophage - mediated tumor cytotoxicity. Macrophages can be activated by endotoxin and various cell mediators and regulators to kill tumor cells by producing tumor necrosis factor (Keller 1990), oxygen free radicals (Nathan 1980) and reactive nitrogen intermediates (Hibbs 1988, Keller 1990). The intent of the present investigation was to ascertain if the pineal indoles, melatonin and methoxytryptamine, could affect the viability of macrophages and stimulate the production of tumor necrosis factor, superoxide, hydrogen peroxide and reactive

nitrogen intermediates by macrophages. The results would shed light on the mechanism of the antitumor effects of pineal indoles in vivo.

3.2 Material and methods

3.2.1 *Chemicals*

Melatonin (MEL), methoxytryptamine (MTA), lipopolysaccharide (LPS) from Escherichia coli, and phorbol 12-myristate-13-acetate-4-O-methylester were obtained from Sigma Chemical Co. Stocks of melatonin and methoxytryptamine were prepared by dissolving 50 mg pineal indole in 2 ml 95% alcohol.

3.2.2 *Culture media*

Dulbecco's modified Eagle's medium without phenol red (catalog no. D 2902), Dulbecco's phosphate buffered saline (catalog no. D 5652), Hank's balanced salts (catalog no. H 2387) and RPMI-1640 (catalog no. R 6504) were purchased from Sigma Co. Generally, they were first unpacked and dissolved in 800 ml fresh double-distilled water. They were stirred gently for 3 - 4 hours. 2 g sodium bicarbonate was then added into the RPMI, 3.7 g sodium bicarbonate was added into the Dulbecco's modified Eagle's medium and 0.35 g sodium bicarbonate was added into the Hank's balanced salt solution. 0.1 g $MgCl_2 \cdot 6\ H_2O$ and 0.133 g $CaCl_2 \cdot 2\ H_2O$ were added into the Dulbecco's phosphate-buffered saline. Each of the solutions was stirred for a few minutes and the pH was adjusted to 7.2 while stirring. Then

3.2.5 *In vitro cytotoxicity of melatonin and methoxytryptamine on mouse peritoneal macrophages*

Peritoneal macrophages from C57 mice were seeded into 96-well culture plates (Nunclon, Denmark) at a concentration of 2×10^5 cells/0.1 ml/well and incubated at 37 °C in a humidified 95 % air - 5 % CO_2 atmosphere for 2 hours. The plate was washed with Hanks' balanced salt solution. Adherent macrophages were incubated with 125, 250 or 500 μM pineal indole for 48 hours. Cells incubated with the medium which possessed the same alcoholic concentration as the solvent for pineal indoles served as the control. After incubation for 24 hours, the culture was washed with phosphate - buffered saline and then incubated with phosphate - buffered saline containing 0.005 % neutral red for 1 hour. The culture was then washed with phosphate buffered saline containing 1 % formaldehyde, 1 % $CaCl_2$ and the remaining medium was drained. After the culture was dried in air, the neutral red phagocytosed by the macrophages was extracted by the lysis buffer which contained 1 % acetic acid - 50 % alcohol. The neutral red uptake of the macrophages was determined using a micro ELISA autoreader (3550 Bio-Rad microplate reader) at 540 nm. Viability of the cells expressed as a percentage was calculated from the ratio of the absorbance of cells treated with pineal indole to the absorbance of control cells treated with culture medium containing 0.38 % alcohol.

3.2.6 *Production and bioassay of tumor necrosis factor-α (TNF-α)*

Adherent peritoneal macrophages in 24-well culture plates (Nunclon, Denmark) at a

concentration of 2 X 10^6 cells/ml/well were obtained from mice treated with pineal indoles. The cells were cultured for 1, 2, and 4 hours in RPMI 1640 containing 1 μg LPS /ml. The supernatant was collected and stored at - 70 °C for the L929 bioassay. 1×10^4 L929 fibroblasts /0.1 ml/well were seated into 96-well culture plates (Corning, USA) and incubated at 37 °C in a humidified 95 % air - 5 % CO_2 atmosphere for 24 hours. The DNA synthesis of the L929 fibroblasts was arrested by incubating the cells with RPMI containing 5 μg actinomycin D (catalog no. A 1410, Sigma) /ml for 2 hours. The L929 cells were then washed by Hanks' balanced salt solution and cultured with different dilutions of TNF-containing medium for 24 hours. After incubation, the plates were washed in phosphate buffered saline and the remaining cells were stained with crystal violet (0.5 % in methanol/water : 1/4 v/v) for 3 minutes. The plates were washed with phosphate buffered saline several times and the medium was decanted. After the culture was dried in air, the macrophages were lysed by lysis buffer which contains 1 % acetic acid - 50 % alcohol. The quantity of crystal violet taken up by the macrophages was determined using a micro ELISA autoreader (3550 Bio-Rad microplate reader USA) at 595 nm.

3.2.7 *Preparation of TNF-α and Actin DNA probe (Sambrook et al., 1989)*

3.2.7.1 *Preparation of the LB agar*

10 g bacto-tryptone, 5 g bacto-yeast extract and 10 g NaCl were dissolved in 800 ml double distilled water. The pH was adjusted to 7.4, 15 g agar was added and the volume was adjusted to 1 L. The solution was autoclaved, cooled down to 50 °C,

stirred gently, poured into 10 cm diameter petri dishes aseptically, and allowed to stand for 2 hours at room temperature before use.

3.2.7.2 *Preparation of LB medium*

10 g bacto-tryptone, 5 g bacto-yeast extract, and 10 g NaCl were dissolved in 800 ml double distilled water. The pH was adjusted to 7.4 and the volume to 1 L. The solution was autoclaved, cooled down at room temperature and then stored at 4 °C.

3.2.7.3 *Preparation of competent cells*

Escherichia coli strain HB 101 cells were spread on LB agar and incubated at 37 °C overnight. One of the colonies was added to 5 ml LB medium and the tube was shaken overnight at 37 °C with a speed of 100 - 200 rpm. 1 ml of the overnight culture was pipetted into 99 ml LB medium and shaken at 37 °C for about 2 hours until the growth of the bacteria was at the log phase of their growth (the absorbance of the mixture at 550 nm was in the range 0.3 - 0.4). The culture was spun (2000 g) at 4 °C for 10 minutes. The supernatant was discarded and the pellet was resuspended in 50 ml ice - cold solution containing 50 mM $CaCl_2$ and 10 mM Tris - Cl at pH 8.0 in order to induce a transient state of "competence" in the recipient bacteria (increase the permeability of the cell wall), during which they were able to take up DNAs derived from a variety of sources. The solution was put on ice for 15 minutes and spun at 4 °C for 5 minutes. The supernatant was discarded and the pellet was resuspended in 6.67 ml ice - cold solution containing 50 mM $CaCl_2$ and

10 mM Tris - Cl at pH 8.0. The competent cells can be stored at 4 °C for 24 hours.

3.2.7.4 *Transformation of plasmid*

0.5 μl of the TNF-α (from Dr. Beutler, Texas) or Actin (from Dr. C. C. Wong, C.U.H.K.) DNA probe containing plasmid, 9.5 μl sterile distilled water, and 240 μl competent cells were added to a 5 ml culture tube and put on ice for 10 minutes. The tube was incubated to 42 °C for 2 minutes and cooled down on ice after incubation. 0.75 ml LB medium was added into the tube after the tube had been cooled down. The tube was then incubated at 37 °C for 1 hour.

3.2.7.5 *Extraction of plasmid*

330 μl of the transformed HB 101 cells carrying the plasmid of TNF-α or Actin DNA insert was spread on LB agar with ampicillin (50 mg ampicillin was added before the warm LB agar solution was poured into 10-cm diameter petri dishes) and incubated at 37 °C overnight. One of the colonies was added to 5 ml LB medium and shaken overnight at 37 °C with a speed of 100 - 200 rpm. The culture was spun (2000 g) at 4 °C for 10 minutes. The supernatant was discarded and the pellet was resuspended in 2.25 ml glucose buffer (25 mM Tris, pH 8.0, 50 mM glucose, and 10 mM EDTA), which was isosmotic solution. 750 μl fresh lysozyme solution (8 mg/ml glucose buffer) was added and incubated for 5 minutes at room temperature. The mixture was transferred to a 15 ml Corex centrifuge tube. 6 ml 0.8 % NaOH - 1 % SDS was added into the tube in order to lyse the resulting spheroplasts to liberate the

plasmid from the pressurized interior of the bacteria. The tube was placed on ice for 5 minutes. 4.5 ml ice-cold potassium acetate solution was added and spun (12000 g) at 4 °C for 10 minutes. The pellet was discarded and the supernatant was transferred into another Corex centrifuge tube. 7.5 ml isopropanol was added into the supernatant and the mixture was mixed by vortexing. The tube was placed in a - 20 °C freezer for 30 minutes. The tube was then spun (12000 g) at 4 °C for 15 minutes. The supernatant was discarded and the pellet was resuspended in 800 µl Tris-EDTA buffer (10 mM Tris•Cl, 1 mM EDTA, pH 7.5). The solution was transferred to two 1.5 ml microfuge tubes. 20 µg RNAase was added into each tube to remove the RNA and the tubes were incubated at room temperature for 15 minutes. The DNA in the solution was then extracted with 1 volume of phenol (equilibrated by 0.1 % dehydroxyquinoline - 1 M Tris at pH 8.0), 2 volumes of phenol - chloroform - isoamyl-alcohol mixture (25 : 24 : 1 by volume) and 0.5 volume of chloroform - isoamy-alcohol (24 : 1 by volume) successively to extract the protein because Escherichia coli strain HB 101 cells was able to express endonuclease A, which degraded plasmid DNA during subsequent incubation in the presence of Mg^{++}. The aqueous phase obtained after each extraction was transferred to a 2 ml microfuge tube. 1/10 volume of 3 M sodium acetate and 2.5 volumes of ice - cold ethanol were added. The DNA in the solution was then precipitated at - 70 °C for 1 hour. After precipitation, the tubes were spun (12000 g) at 4 °C for 10 minutes. The solution was discarded and the pellet was resuspended in 1 ml 70 % alcohol. The tube was spun (12000 g) at room temperature for 3 minutes. The alcohol was discarded and the pellet was lyophilized for 10 minutes. The pellet was resuspended in 10 µl Tris-EDTA (pH 7.5).

3.2.7.6 *Separation of the DNA probe*

The extracted DNA was subjected to enzyme digestion. 0.5 volume of Pst I (catalog no. 140S, New England Biolab, USA) and 0.5 volume of NEBuffer 3 (supplied with Pst I as 10 X concentration stock), 0.5 volume of BamH I (catalog no. 136S, New England Biolab, USA) and 0.5 volume of NEBuffer BamH I(supplied with BamH I as 10 X concentration stock) and 7 volumes of double - distilled water were used to digest the DNA sample carrying the TNF-α DNA probe insert. Pst I alone was used to digest the DNA sample carrying the actin DNA probe insert. The digestion was carried out at 37 °C for at least 2 hours. Then the digested DNA was subjected to TAE agarose gel electrophoresis. 193.6 g Tris base, 108.9 g $CH_3COONa \bullet 3\ H_2O$, and 12.5 g EDTA-$Na_2 \circ 2\ H_2O$ were dissolved in double - distilled water and the pH was adjusted to 7.2 with acetic acid and the volume to 1 L to serve as 40 X TAE. 9 volumes of double distilled water and 1 volume of loading buffer (5 ml glycerol, 250 µl 40 X TAE, 1 ml saturated bromophenol blue, 2.75 ml double distilled water were added together to make a 10 ml stock) were mixed with the extracted DNA and the mixture was incubated at 65 °C for 2 minutes. It was then loaded onto TAE agarose gel (prepared by dissolving 0.5 g agarose in the mixture of 1 ml 50 X TAE, 5 µl ethidium bromide, 49 ml double - distilled water) using 1 X TAE as running buffer. Gel electrophoresis was run at 100 V until the front of bromophenol blue reached the positive pole. Because the size of the TNF-α DNA insert was about 1.3 kb and the size of the actin DNA insert was about 1.8 - 2 kb, Lambda DNA BstE II was used as the marker DNA.

3.2.7.7 *Purification of the DNA probe*

After electrophoresis, the gel (only the area of the correspondent size) was cut and subjected to DNA elution using Geneclean®II kit (Bio 101 Inc., catalog no. 3106). It was weighed and its volume was deduced by assuming that 1 g of the gel occupied a volume of about 1 ml. The cut gel was placed in a 2-ml microfuge tube and solubilized with 3 volumes of NaI (5.3 M so that the final concentration of NaI was above 4 M). The tube was incubated at 50 °C for 2 minutes. The tube was mixed thoroughly and incubated at 50 °C for 5 minutes. The gel was thus dissolved and 10 µl glassmilk (provided in the Geneclean®II kit, generally 5 µl for 5 µg DNA, then 1 µl for each additional 0.5 µg DNA, but not harmful in excess) was added. The solution was mixed gently by pipetting up and down. The tube was put on ice for 5 minutes to allow binding of DNA to the silica matrix. The mixture should be mixed every 1 - 2 minutes. The tube was spun for a while. The supernatant was better transferred to another microfuge tube for storage so that it could be restored by another method when no DNA binding was found. The pellet was washed three times with 200 - 700 µl ice - cold NEW WASH (Bio 101, catalog no. 3109). The tube was spun for a while. The supernatant was discarded and the pellet was resuspended in Tris-EDTA buffer (same volume as that of glassmilk added). The mixture was incubated at 50 °C for 3 minutes and spun for 30 seconds. The supernatant was collected and the pellet was washed once more with Tris-EDTA buffer. The supernatant was collected. The purified DNA was stored at - 20 °C and was ready for hybridization.

3.2.8 *Northern blotting technique for hybridization*

3.2.8.1 *Isolation of total RNA*

Total RNA was extracted according to the method of Chirgwin et al (1979). Briefly, 4 X 10^8 peritoneal macrophages /10 ml/plate, similarly stimulated by LPS, were cultured in a 10-cm diameter culture dish (Falcon) for extraction of total cellular RNA after 0.5, 1 and 2 hours. The cells were lysed using 3 ml of 50 mM Tris buffer (pH 7.5) containing 5 M guanidinium thiocyanate, 10 mM EDTA and 8 % 2 - mercaptoethanol. 21 ml of 4 M lithium chloride was added to protect the RNA from RNAase. The lysate was incubated at 4 °C overnight and then spun (10000 g) (Beckman) at 4 °C for 1 hour. The pellet was resuspended in 5 ml of 3 M lithium chloride and spun (10000 g) at 4 °C for 20 minutes. The pellet was resuspended in 1 ml solubilization buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 0.1 % SDS). The mixture was frozen in liquid nitrogen and vortexed while thawing. The mixture was then transferred to a 2-ml microfuge tube and extracted with 0.7 - 0.8 ml phenol. It was spun (12000 g) at room temperature for 3 minutes. The aqueous phase was transferred into another tube and extracted with 0.7 - 0.8 ml phenol-chloroform-isoamyl alcohol mixture (25:24:1 by volume). It was spun (14000) rpm at room temperature for 3 minutes. The aqueous phase was transferred into another tube and extracted with 0.7 - 0.8 ml chloroform - isoamyl alcohol (24:1 by volume). The tube was spun (12000 g) at room temperature for 3 minutes. The aqueous phase was transferred into another tube and 0.1 ml 5 M NaCl and 0.9 ml isopropanol were added to the aqueous phase. The tube was then left at - 70 °C for 1 hour. The tube

was spun (12000 g) at 4 °C for 10 minutes. The solution was discarded and the pellet was resuspended in 1 ml 70 % alcohol. The tube was spun (12000 g) at room temperature for 3 minutes. The alcohol was discarded and the pellet was lyophilized for 10 minutes. The pellet was resuspended in 10 µl Tris-EDTA (pH 7.5). The quantity of RNA was then determined by measuring the absorbance at 260 nm.

3.2.8.2 *Separation of RNA*

The isolated total RNA was then loaded on 1 % formaldehyde gel for electrophoresis. The 1 % formaldehyde gel was prepared by dissolving 0.5 g agarose in 36.08 ml DEP H_2O (prepared by autoclaving a 0.1 % diethylpyrocarbonate solution, boiling it to expel the DEPC until there was no smell of DEPC), 5 ml 10 X MOPS/EDTA (41.8 g morpholinopropane-sulfonic acid, 6.8 g sodium acetate, and 3.8 g tetrasodium EDTA salt were dissolved in 1 L double distilled water, pH 7.0), 8.93ml formaldehyde, and 5 µl ethidium bromide. The running buffer was 250 ml 1 X MOPS/EDTA. The loading buffer was a mixture of 150 µl 10-fold dye (50 % glycerol + 1 mM EDTA, pH 7.4 + 0.4 % bromophenol blue), 150 µl 10-fold MOPS/EDTA, 255 µl 37 % formaldehyde, 750 µl formamide. 6.67 volumes of loading buffer was mixed with 20 µg RNA. It was incubated at 65 °C for 2 minutes and cooled down in ice. It was spun down and loaded into the wells of the 1 % formaldehyde gel. It was then run overnight at 12 V from the negative pole to the positive pole.

3.2.8.3 *Northern blotting*

The gel was examined under an ultraviolet illuminator to visualize the separation. 18 S and 28 S RNA should be seen. The left corner of the first lane was cut for marking the location of the first lane. The gel was then ready for blotting. A Zeta Probe membrane (Amersham) with a size similar to that of the gel was first soaked in distilled water and then in 10 X SSC (87.65 g NaCl, 44.1 g sodium citrate in 1 L double distilled water, pH 7.0, sterilized) for 30 minutes. Two filter papers (3 mm) were used to prepare the paper bridge by soaking in 10 X SSC. The soaked filter papers were put on the plastic bridge which was located on top of a tank containing 200 ml 10 X SSC. The gel was put upside down on the paper bridge. The soaked Zeta Probe membrane was cut at one of the corners and put on the gel so that the cut corner of the membrane matched the cut corner of the gel. The tank was then wrapped by cling wrap to avoid evaporation of 10 X SSC. The edge of the cling wrap should be folded for more powerful isolation. Four filter papers were cut to a size similar to that of the gel and 2 of them were soaked in 10 X SSC. The 2 wet filter papers were put on the Zeta Probe membrane first and 2 dry filter papers were put on top of the deck. A deck of paper towel was added on the deck. A 500 g weight was put on top of the deck to induce a capillary force which made the 10 X SSC move on to the filter paper, pass through the gel, and conduct the content of the gel to move toward the membrane. After overnight transfer, the gel and the blot were examined under a ultraviolet illuminator to see whether the transfer was complete. The blot was washed in 50 ml 2 X SSC for 30 minutes. The blot was cross - linked at 0.12 J/cm^2 by a ultraviolet cross - linker (Spectrolinker XL 1500 UV Crosslinker, Spectronics Corporation) 2 times. The blot was then wrapped by two filter papers in the plastic bag for storage at room temperature.

3.2.9 *Hybridization of TNF-α mRNA and Actin mRNA*

3.2.9.1 *Prehybridization*

The buffer used for pre-hybridization was FSSSE (7 g SDS in 100 ml mixture containing 50 ml of 100 % formamide, 25 ml of 1 M $NaHPO_4$ at pH 7.2, 5 ml of 5 M NaCl, and 0.5 ml of 0.5 M EDTA at pH 8.0, 19.5 ml double distilled water). The blot was put into a bottle and 5 ml FSSSE was pipette into the bottle. The bottle was put into a rotating incubator which was maintained at 42 °C so that each part of the blot could be soaked thoroughly. After overnight pre-hybridization, the blot was ready for hybridization.

3.2.9.2 *Probe labelling*

The TNF-α probe and actin probe were labelled with ^{32}P-dCTP by random priming. 2 µl TNF-α DNA probe containing 100 ng DNA was added into an eppendorf tube. 5 µl double-distilled water and 1.25 µl 0.1 U/µl hexamer (Pharmacia 27-2166-01 random primers) were then added. The mixture was then boiled for 2 minutes and transferred to ice immediately. It was spun down by brief centrifugation at 3000 rpm. 1 µl bovine serum albumin (10 mg/ml), 5 µl ^{32}P-dCTP, 0.75 µl klenow fragment (5000 U/ml, Pharmacia 27-0928-02) and 10 µl reaction buffer (mixture of 0.5 M Hepes at pH 6.6, 12.5 mM $MgCl_2$, 0.025 M ß - mercaptoethanol, 0.125 M Tris pH 8.0, 50 µM dATP and 50 µM dGTP, 50 µM dTTP) were added, bringing the total volume to 25 µl. The reaction was then allowed to proceed at 37 °C for 3 hours.

A Sephadex G50 gel column was packed in a 1-ml syringe before the reaction was completed. After completion of reaction, the reaction mixture was loaded onto the column with 150 µl 10+1 TE - 0.1 % SDS (mixture of 5 ml of 2 M Tris at pH 7.5, 0.4 ml of 0.25 M EDTA, 1 ml of 10 % SDS and 93.6 ml of double distilled water). It was spun down and collected in an eppendorf tube which was put into the test tube before loading. 1 µl reaction mixture was then pipetted into an empty scintillation counting vial. The radioactivity of the labelled probe was then measured with a Beckman ß-counter. The amount of the labelled probe used was about 1 million cpm per lane.

3.2.9.3 *Hybridization*

The pre-hybridization buffer in the bottle was replaced with 5 ml fresh FSSSE after rolling at 42 °C. A suitable amount of ^{32}P-dCTP labelled TNF-α or actin DNA probe was added into the bottle. The hybridization mixture was then rolled at 42 °C overnight.

3.2.9.4 *Autoradiography*

After complete hybridization, the blot was washed with 0.1 % SDS - 2 X SSC solution 3 times. The blot was transferred to room temperature and shaken in 0.1 % SDS - 2 X SSC solution at a speed of 236 rpm for 15 minutes. The blot was then shaken in 0.1 % SDS - 0.2 X SSC solution for 15 minutes. The blot was finally shaken twice in 0.1 % SDS - 0.1 X SSC solution at 37 °C for 15 minutes. A low

count of the blot was preferred because the non - specific binding can always be washed away. If the count was still high, increasing the temperature should help to solve the problem. The washed blot was wrapped using saran wrap. The wrapped blot was then exposed to a Kodak XAR - 5 film at - 70 °C for 1 day. After the film was exposed, it was developed in developer (5-fold dilution of developer of Kodak, Australia, catalog no. 405 1298) for 5 minutes, rinsed with water, fixed in fixer (4-fold dilution of liquid x-ray fixer and replenisher of Kodak, Australia, catalog no. 405 1611) for 5 minutes, rinsed with water and then air - dried. The autoradiogram was scanned with a desitometer (GS 3000 Transmittance/Reflectance Scanning Desitometer, Hoefer Scientific Instruments) to compare the band intensities. The blot was then shaken in 0.1 % SDS - 0.1 X SSC solution at 95 °C for 15 - 30 minutes to wash away the DNA probe. The washed blot was then ready for other hybridizations.

3.2.10 *Assay for nitrite production by peritoneal macrophages*

Nitrite is a stable metabolite of reactive nitrogen intermediate. The Griess reaction is a specific and sensitive test for nitrite (Hibbs 1988). Nitrite reacts in acid solution, by intermediate formation of nitrous acid, with primary aromatic amines (for example, naphthalene-ethylenediamine) form diazonium compounds which are able of coupling with amino or hydroxy derivatives (for example, sulfanilamide) to form azo dyes (for example, naphthalene-ethylenediamine-p-azobenzene-sulphonic acid). Thioglycolate - elicited peritoneal macrophages were collected and washed with Hanks' balanced salt solution. They were resuspended at a concentration of 2 X 10^6

cells/ml supplemented DMEM without phenol red. The cells were allowed to adhere onto the surface of a 96 - well culture plate for 2 hours before they were challenged with lipopolysaccharide (1 μg/ml) for 1 day. The supernatant was then allowed to react with half its volume of Griess reagent (1 % sulfanilamide in 5 % H_3PO_4 - 0.1 % naphthalene-ethylenediamine dihydrochloride) for 15 minutes and the nitrite present in the culture medium was measured with an ELISA reader at 540 nm. The concentration of nitrite was estimated using $NaNO_2$ as the standard and expressed in μM.

3.2.11 *Assay for production of reactive oxygen intermediate (superoxide anion) by peritoneal macrophages*

The method is the reduction of ferricytochrome c by O_2^-, a process in which O_2^- serves as an electron donor (Edgar 1981). Amount of reduced cytochrome C is determined by measuring its absorbance at 550 nm. Peritoneal macrophages at a concentration of 2 X 10^6 cells/ml were cultured in phosphate buffered saline supplemented with 1 g glucose/L and 80 μM cytochrome C (Sigma C2506, Type III) and stimulated simultaneously with 200 nM phorbol 12-myristate-13-acetate-4-O-methylester (Sigma, catalog no. P8139). The cells were incubated at 37 °C for 1 hour. The reaction was stopped by placing the mixture in ice. The amount of superoxide anion released into the culture medium was calculated from the extinction coefficient by the formula $\Delta_{550\,nm}$ x light path (cm) = 21 x 10^3 M^{-1} and the superoxide concentration was expressed in μM.

3.2.12 *Assay for production of hydrogen peroxide by peritoneal macrophages*

The method is based on the horseradish peroxidase-dependent oxidation of phenol sulfonphthalein, which is also known as phenol red (Edgar 1981). The composition of PRS (phenol red solution) assay medium is 8.18 g/L NaCl, 0.99 g/L glucose, 0.2 g/L phenol red, 19000 U/L horseradish peroxidase (Sigma P-8125, Lot 15F-8520), and 10 mM potassium phosphate buffer at pH 7.0 (0.136 g KH_2PO_4 and 0.276 g $Na_2HPO_4 \cdot H_2O$ dissolved in 200 ml double distilled water with the pH adjusted to 7.0 and the volume to 255 ml). Peritoneal macrophages at a concentration of 2 X 10^6 cells/ml were cultured in PRS and stimulated with 200 nM phorbol 12 - myristate 13 - acetate 4 -O - methylester at 37 °C for 1 hour. The reaction was stopped by addition of 1/10 volume (10 µl) of 1 N NaOH. The absorbance at 600 nm was measured. The concentration of H_2O_2 was calculated using serial dilutions of 30 % H_2O_2 (Merck Art. 8597) as standard and expressed in µM.

3.2.13 *Treatment of peritoneal macrophages with pineal indole <u>in vitro</u> followed by measurement of production of nitrite, superoxide or hydrogen peroxide by peritoneal macrophages in vitro*

Adherent peritoneal macrophages were incubated with melatonin or methoxytryptamine at a concentration of 0.5, 5, or 50 µM for 48 hours. The culture medium was decanted and the macrophages were washed with phosphate buffered saline. The macrophages were then stimulated with lipopolysaccharide (1 µg/ml for 1 day) or phorbol 12-myristate-13-acetate-4-O-methylester (200 nM for 1 hour). In

the case of lipopolysaccharide stimulation, Griess reagent was added to react with nitrite present in the culture medium. In the case of phorbol ester stimulation, the culture medium was taken for superoxide measurement or for hydrogen peroxide determination after addition of 1 N NaOH to terminate the reaction as detailed above.

3.2.14 *Statistics*

The data were analyzed by one way analysis of variance using Scheffe's multiple range test. The levels of significance were taken at $p < 0.05$.

3.3 Results

3.3.1 *In vitro effects of melatonin and methoxytryptamine on viability of peritoneal macrophages from C57 mice*

Adherent peritoneal macrophages from normal C57 mice were incubated with melatonin or methoxytryptamine at a concentration of 125, 250, 500 μM for 48 hours. The cell viability is shown in Figure 3.1. Although slight decreases in cell viability were found when the dose of melatonin or methoxytryptamine was increased, about 95 % of the cells were still viable at the highest concentration of pineal indoles tested. Thus melatonin and methoxytryptamine exerted only a slight cytotoxic effect on normal peritoneal macrophages at high concentrations.

3.3.2 *In vitro effects of melatonin and methoxytryptamine on nitrite production by*

peritoneal macrophages from C57 mice

Melatonin or methoxytryptamine at a concentration of 0.5, 5, or 50 μM was incubated with adherent peritoneal macrophages for 48 hours. The concentration of nitrite released into the culture medium was calculated from the $NaNO_2$ standard curve (Figure 3.2). Melatonin at 5 μM and methoxytryptamine at 0.5 μM were found to enhance nitrite production (24 μM and 30 μM respectively) (Figure 3.3). Both melatonin and methoxytryptamine exerted a smaller enhancing effect on nitrite production when their concentration was increased. The nitrite level returned to the control level when the indole concentration was increased to 50 μM. The concentration of nitrite produced by stimulated peritoneal macrophages of control mice was about 16 μM.

3.3.3 *In vitro nitrite production by peritoneal macrophages from C57 mice which had been treated with melatonin or methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks*

Peritoneal macrophages isolated from melatonin - and methoxytryptamine - treated mice produced a higher level of nitrite when compared to the control (Figure 3.4). Melatonin - treated mice produced a 2 - fold higher level of nitrite than the control group. Methoxytryptamine - treated mice produced only a slightly higher nitrite level than the control group. The concentration of nitrite produced by stimulated peritoneal macrophages from control mice was about 8 μM.

3.3.4 *In vitro effects of melatonin and methoxytryptamine on hydrogen peroxide production by peritoneal macrophages from normal C57 mice*

The concentration of hydrogen peroxide released into the culture medium was calculated from the H_2O_2 standard curve (Figure 3.5). The results are presented in Figure 3.6. Hydrogen peroxide production decreased, although not significantly, when the dose of melatonin was increased. The production of hydrogen peroxide decreased by about 50 % in the presence of 0.5 μM methoxytryptamine and by about 60 % when the dose of methoxytryptamine was increased to 50 μM. The decrease in production of hydrogen peroxide in response to methoxytryptamine treatment was significant. The concentration of hydrogen peroxide produced by the stimulated peritoneal macrophages in the control was about 1.4 μM.

3.3.5 *In vitro hydrogen peroxide production by peritoneal macrophages from C57 mice which had been treated with melatonin or methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks*

The results are presented in Figure 3.7. Peritoneal macrophages isolated from melatonin - treated mice produced more hydrogen peroxide but the increase was not significant. Peritoneal macrophages isolated from methoxytryptamine - treated mice produced less (about 40 % less than the control level) hydrogen peroxide. The concentration of hydrogen peroxide produced by peritoneal macrophages from the control mice was about 2 μM.

3.3.6 *In vitro effects of melatonin and methoxytryptamine on superoxide production by peritoneal macrophages from C57 mice*

The results are presented in Figure 3.8. Melatonin and methoxytryptamine at a concentration of 0.5 μM increased superoxide production by about 15 %. No significant changes were produced at higher concentrations of both pineal indoles. The concentration of superoxide produced by stimulated peritoneal macrophages from control mice was about 4.5 μM.

3.3.7 *In vitro superoxide production by peritoneal macrophages from C57 mice which had been treated with melatonin or methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks*

The results are presented in Figure 3.9. Peritoneal macrophages isolated from melatonin - treated mice produced 20 % more superoxide than those from control mice and the difference was significant. Peritoneal macrophages isolated from methoxytryptamine - treated mice produced 30 % less superoxide than cells of control mice and the difference was also significant. The concentration of superoxide produced by stimulated peritoneal macrophages from control mice was about 12.5 μM.

3.3.8 *In vitro effects of melatonin and methoxytryptamine (50 μM) on production of tumor necrosis factor-α by peritoneal macrophages from C57 mice*

The results are presented in Figure 3.10. The L929 bioassay is a sensitive test for tumor necrosis factor-α. The larger the amount of tumor necrosis factor-α, the greater is the inhibition of growth of L929 cells. The secretion of tumor necrosis factor-α by lipopolysaccharide - stimulated macrophages increased as the duration of stimulation increased from 1 hour to 4 hours. The tumor necrosis factor-α containing supernatant was diluted 200 folds for assay. Generally speaking, there was little or no difference in the ability to produce tumor necrosis factor-α between macrophages from control mice and those from melatonin - treated or methoxytryptamine - treated mice. Although there was less tumor necrosis factor-α produced by macrophages of methoxytryptamine - treated mice when compared to those from control mice after 4 hours of LPS stimulation, the difference was only about 6 %.

3.3.9 *In vitro production of tumor necrosis factor-α by peritoneal macrophages from C57 mice which had been treated with melatonin or methoxytryptamine in drinking water (100 μg/ml) for 2 weeks.*

The tumor necrosis factor-α-containing supernatant was diluted 200, 400, 800 folds for assay. The results are presented in Figure 3.11, 3.12, and 3.13 respectively. The level of tumor necrosis factor-α was found to be higher (less than 10 % difference in both cases) in macrophages from melatonin - treated or methoxytryptamine - treated mice than those from control mice after 4 hours of LPS stimulation using a dilution of 1 : 200 for the supernatant (Figure 3.11). The level of tumor necrosis factor-α was found to be higher (about 10 % and 5 % respectively) in macrophages from melatonin - treated or methoxytryptamine - treated mice than those from control

mice after 4 hours of LPS stimulation using a dilution of 1 : 400 for the supernatant (Figure 3.12). The level of tumor necrosis factor-α was found to be higher (about 1 fold after 2 hours and about 1.5 folds after 4 hours in the case of melatonin - treated macrophages, and about 1.8 folds after 2 hours, about 2 folds after 4 hours in the case of methoxytryptamine - treated macrophages) in macrophages from melatonin - treated or methoxytryptamine - treated mice than those from control mice after 2 and 4 hours of LPS stimulation using a dilution of 1 : 800 for the supernatant (Figure 3.13).

3.3.10 *Ratio of tumor necrosis factor-α (TNF-α) messenger RNA to actin messenger RNA in macrophages from C57 mice which had been treated with melatonin or methoxytryptamine in the drinking water for 2 weeks after treatment with lipopolysaccharide (LPS) for different durations.*

The result of Northern blot hybridization is shown in Figure 3.14 and Figure 3.15. The ratio of TNF-α mRNA to actin mRNA increased when the duration of lipo - polysaccharide stimulation was increased from 30 minutes to 120 minutes. Macrophages from melatonin - treated mice possessed a 25 % higher TNF-α mRNA level than macrophages from control mice after 30 minutes of LPS stimulation and a slightly lower TNF-α mRNA level than macrophages from control mice after 60 and 120 minutes of LPS stimulation. Macrophages from methoxytryptamine - treated mice exhibited a slightly lower TNF-α mRNA level than macrophages from control mice after 60 minutes of LPS stimulation and a slightly higher TNF-α mRNA level than macrophages from control mice after 120 minutes of LPS stimulation.

3.4 Discussion

The existing literature points to an immunostimulatory effect of melatonin. Jankovic et al (1970) observed that pinealectomy induced an impairment of immune potential. Administration of the ß - adrenergic antagonist, propranolol, which inhibits melatonin synthesis, ensued in diminished primary and secondary humoral responses (Maestroni and Pierpaoli, 1987).

The present study showed that there was a correlation between the actions of melatonin on the production of hydrogen peroxide and nitrite by peritoneal macrophages in vivo and in vitro. A similar observation was also made regarding the effect of methoxytryptamine on generation of hydrogen peroxide, nitrite and superoxide. However, a discrepancy was detected between the in vivo and in vitro actions of melatonin on tumor necrosis factor-α production and those of methoxytryptamine on production of tumor necrosis factor-α and superoxide. The discrepancy noted in the case of tumor necrosis factor-α production was probably due to the fact that in the in vivo study the macrophages had been primed or stimulated for a longer duration by lymphokines produced in response to pineal indole and they subsequently demonstrated a greater response to the stimulant in vitro. The discrepancy seen in the effect of methoxytryptamine on superoxide production is more difficult to explain.

There were similarities and differences between the actions of melatonin and methoxytryptamine on peritoneal macrophages. Both indoles enhanced nitrite

production in vivo and in vitro, superoxide production in vitro and tumor necrosis factor-α production in vitro. On the other hand, methoxytryptamine manifested a distinct suppressive action on hydrogen peroxide production under both in vivo and in vitro conditions whereas melatonin did not evoke a significant change under either condition. Furthermore, while melatonin increased superoxide production in vivo, methoxytryptamine produced a change in the opposite direction. How these differences in actions between the two pineal indoles are related to their structural differences remain to be elucidated in a more extensive investigation in the future. The lack of an adverse effect of melatonin and methoxytryptamine on the viability of peritoneal macrophages supports the meaningfulness of the data obtained in the present study.

Ding (1988) reported that incubation of macrophages with γ-interferon for 48 hours in the presence of lipopolysaccharide inhibited hydrogen peroxide production but augmented nitrite release. Similarly, in the present study methoxytryptamine treatment evoked an increase in nitrite production and a decrease in hydrogen peroxide production, and melatonin treatment elicited a rise in nitrite production but did not produce a marked change in hydrogen peroxide. Ding (1988) made three suggestions with references to his findings. Firstly, γ-interferon was the only cytokine tested able to induce both hydrogen peroxide and nitrite release. Secondly, the secretion of hydrogen peroxide and the secretion of nitrite involve two independent pathways. Thirdly, combination of lipopolysaccharide and α/ß/γ - interferon or tumor necrosis factor-α/ß and γ-interferon can induce nitrite secretion synergistically. Hibbs (1987) proposed that arginine deiminase converted L - arginine

into L - citrulline and ammonia. Ammonia was then oxidized to form nitrite. Stuehr (1989) claimed that reactive radical nitric oxide, which was produced during metabolism of L - arginine to NO_2^-/NO_3^-, was the mediator of macrophage - induced cytostasis. Keller (1990) proved that reactive nitrogen intermediates were the main effectors against tumor necrosis factor-α resistant tumor cells. Keller (1990) suggested that the mechanisms of tumoricidal activities of activated macrophages were reliant on the type of tumor cells and the pathway of macrophage activation. The ability of melatonin and methoxytryptamine to elevate nitrite production by peritoneal macrophages is thus in line with the in vitro cytotoxic action of the indoles on tumor cells.

A sharp increase in oxygen consumption was found after stimulation of phagocytes (Babior 1978). Sbarra and Karnovsky (1959) showed that oxygen consumed in the respiratory burst of phagocytes was not used for energy production since inhibitors of mitochondrial respiration did not decrease oxygen consumption. Babior (1978) suggested that the oxygen consumed was reduced to superoxide by NADPH oxidase which in turn oxidized NADPH to $NADP^+$. The increase in $NADP^+$ led to an activation of the hexose monophosphate shunt which subsequently reduced $NADP^+$ to NADPH which serves as the electron donor of oxygen. Superoxide dismutase then converted the superoxide into hydrogen peroxide. Useless hydrogen peroxide was used to oxidize the reduced glutathione by glutathione peroxidase. Oxidized glutathione was then reduced by glutathione reductase which used NADPH as the electron donor. The glutathione peroxidase - glutathione reductase system hence provided more $NADP^+$. Superoxide and hydrogen peroxide can break the bonds in

proteins, lipids and nucleic acids and oxidize various bonds such as disulfide bonds in enzymes (Nathan 1980). The ability of melatonin to elevate superoxide production by peritoneal macrophages was less obvious while superoxide production was lowered after methoxytryptamine treatment. A similar observation was made regarding the effects of melatonin and methoxytryptamine on hydrogen peroxide production. Superoxide and hydrogen peroxide hence appear to contribute little to the enhancement of tumoricidal activity of peritoneal macrophages by pineal indoles.

Another important effector was tumor necrosis factor-α. Beutler (1991) reported the tumor necrosis factor-α gene has been silenced in non - macrophage cell lines. Tumor necrosis factor-α has been reported to cause prolonged inhibition of tumor growth in the absence of T - cell immunity without causing severe weight loss or rapid development of resistance of the tumor to the growth inhibitory effects of tumor necrosis factor-α (Teng 1991). Kronke (1990) pointed out that tumor necrosis factor-α has been reported to induce genes encoding soluble factors such as collagenase, prostaglandin E_2, granulocyte-macrophage colony stimulating factor, interleukin-6, and tumor necrosis factor-α itself. Tumor necrosis factor-α also enhances the expression of the human leukocyte antigen - A, B, C and D and the receptor of interleukin-2. Tumor necrosis factor-α induces c-myc, c-fos, c-jun which are genes encoding nuclear antigens. Tumor necrosis factor-α inhibits c-myc expression in some tumor cell lines sensitive to the growth inhibitory action of tumor necrosis factor-α. Reid (1989) suggested that there were at least 2 mechanisms of tumor necrosis factor-α mediated cell killing. Cycloheximide is well known to enhance tumor necrosis

factor-α - mediated cell killing in culture. Reid found that using a combination of tumor necrosis factor-α and cycloheximide to treat tumor cell lines yielded 2 different results. Some of them were susceptible to the action of the combination. Others were resistant to the combination and were susceptible to tumor necrosis factor-α only. Strieter (1989) suggested that interleukin-2 was important for the induction of the tumor necrosis factor-α gene expression by both normal peritoneal and alveolar macrophages.

In the present investigation, the observation of an augmented production of tumor necrosis factor-α mRNA and tumor necrosis factor by macrophages from mice treated with melatonin and methoxytryptamine is consistent with the finding of in vitro cytotoxic action of melatonin and methoxytryptamine against tumor cell lines. Thus it appears that the pineal indoles can combat tumor cells directly as well as indirectly via an action on macrophages. An immunostimulatory role of methoxytryptamine is disclosed in addition to a similar function of melatonin which has also been suggested by Jankovic et al (1970) and Maestroni and Pierpaoli (1987).

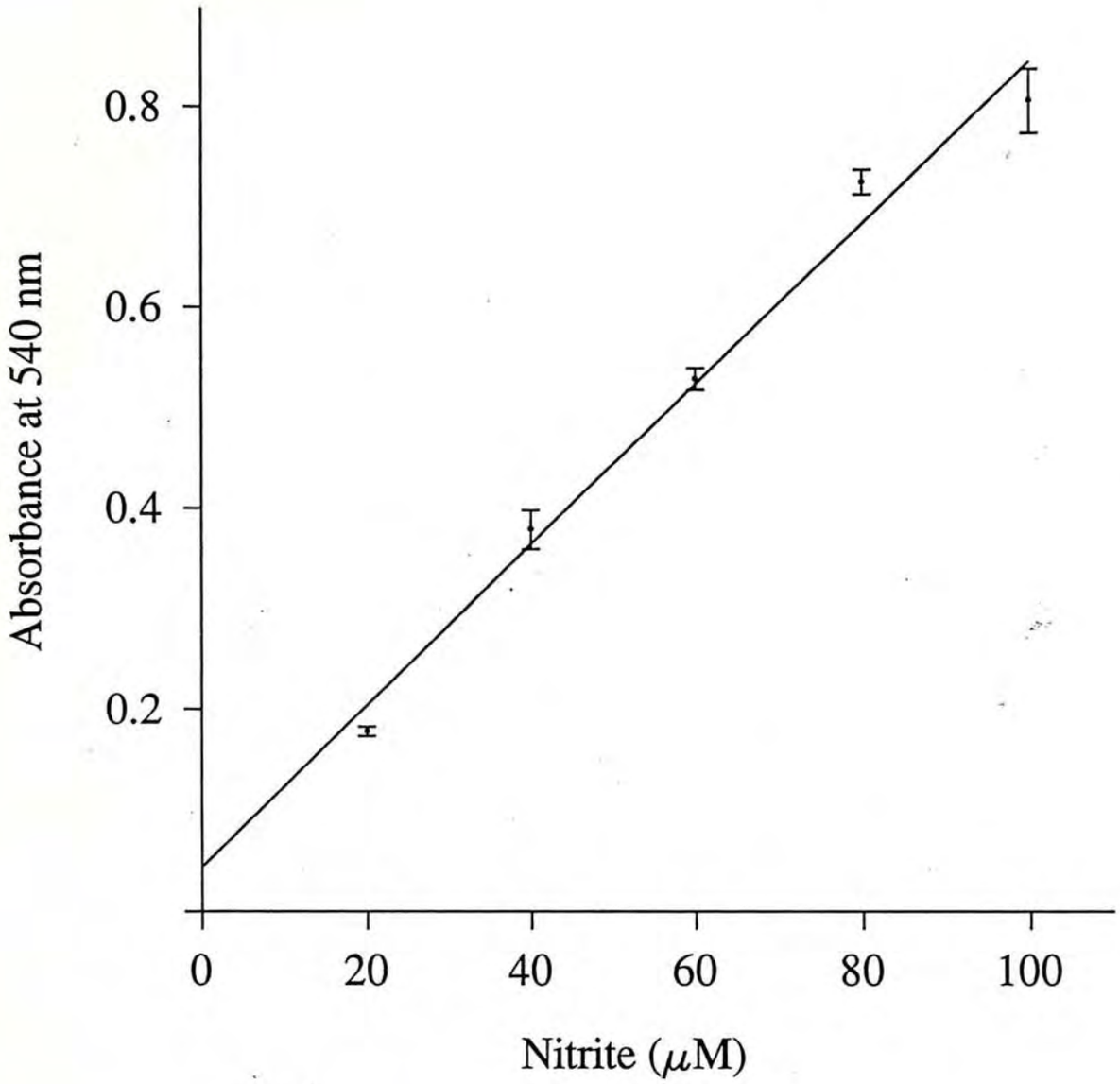


Figure 3.2 Standard curve for nitrite assay using sodium nitrite as standard

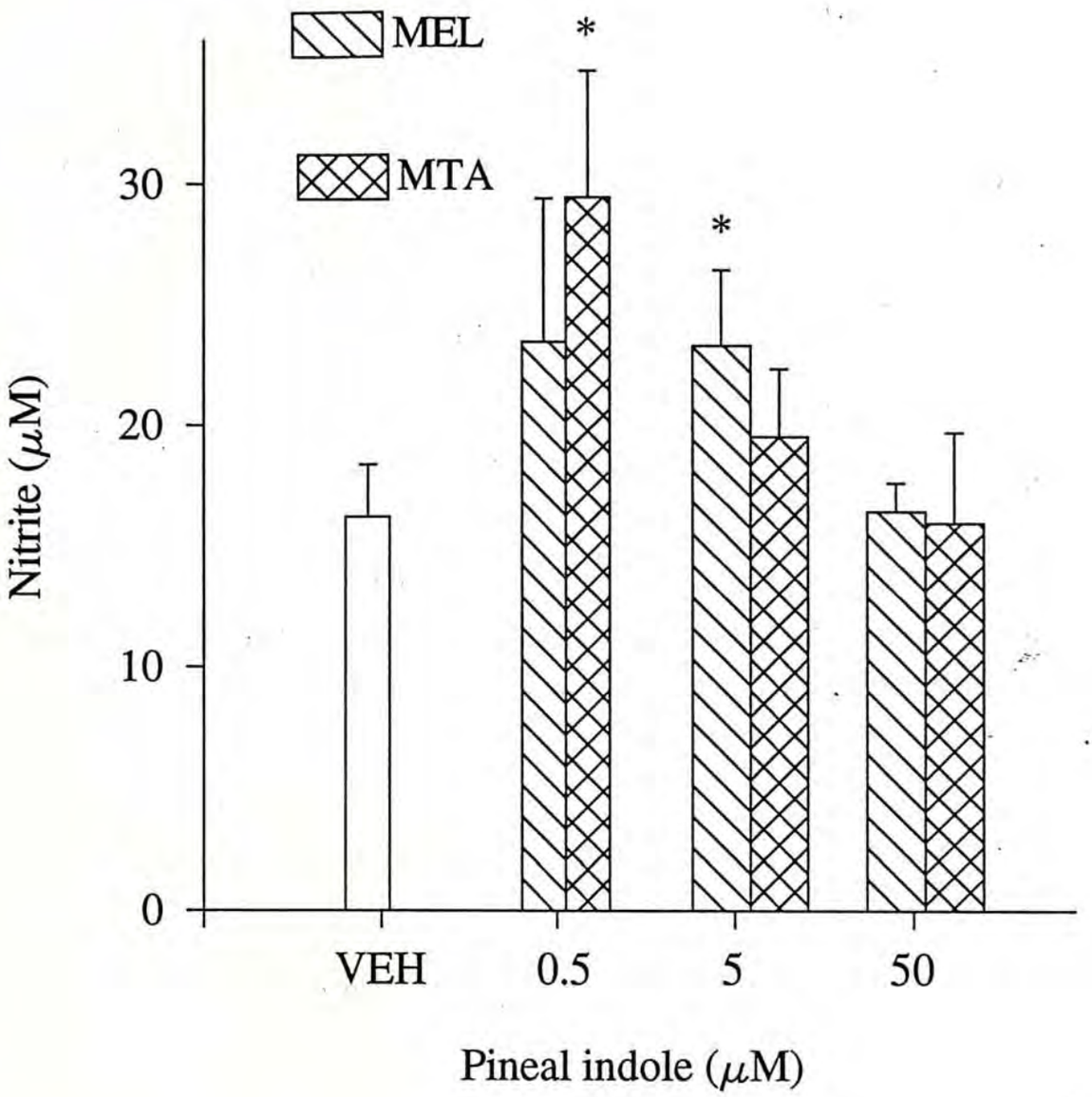


Figure 3.3 In vitro effects of melatonin and methoxytryptamine on nitrite production by peritoneal macrophages from C57 mice. Results are expressed as means ± standard deviation (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

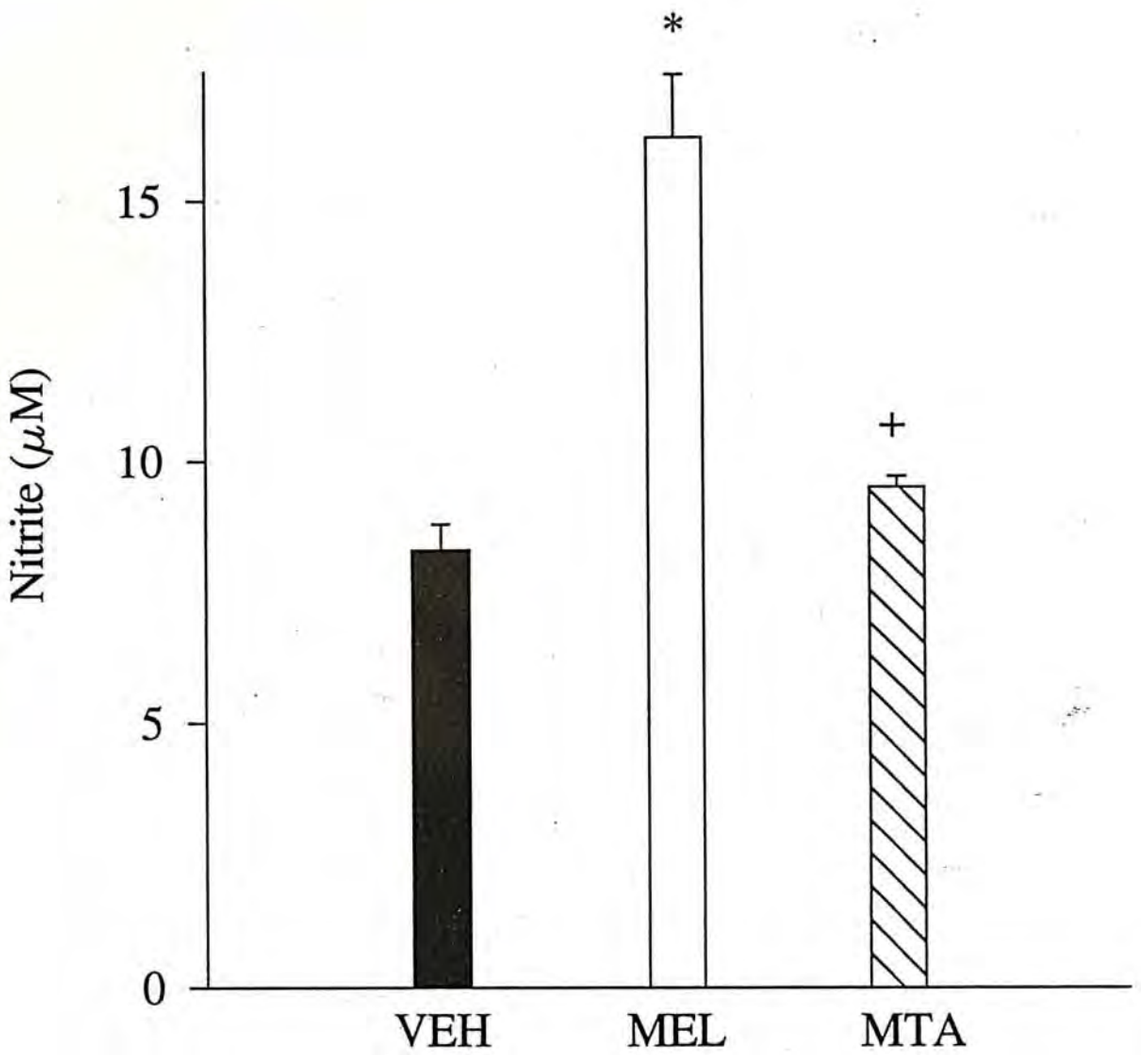


Figure 3.4 In vitro nitrite production by peritoneal macrophages from C57 mice (N = 20/group) treated with melatonin or methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks. Results are expressed as means ± standard deviation (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from melatonin group. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

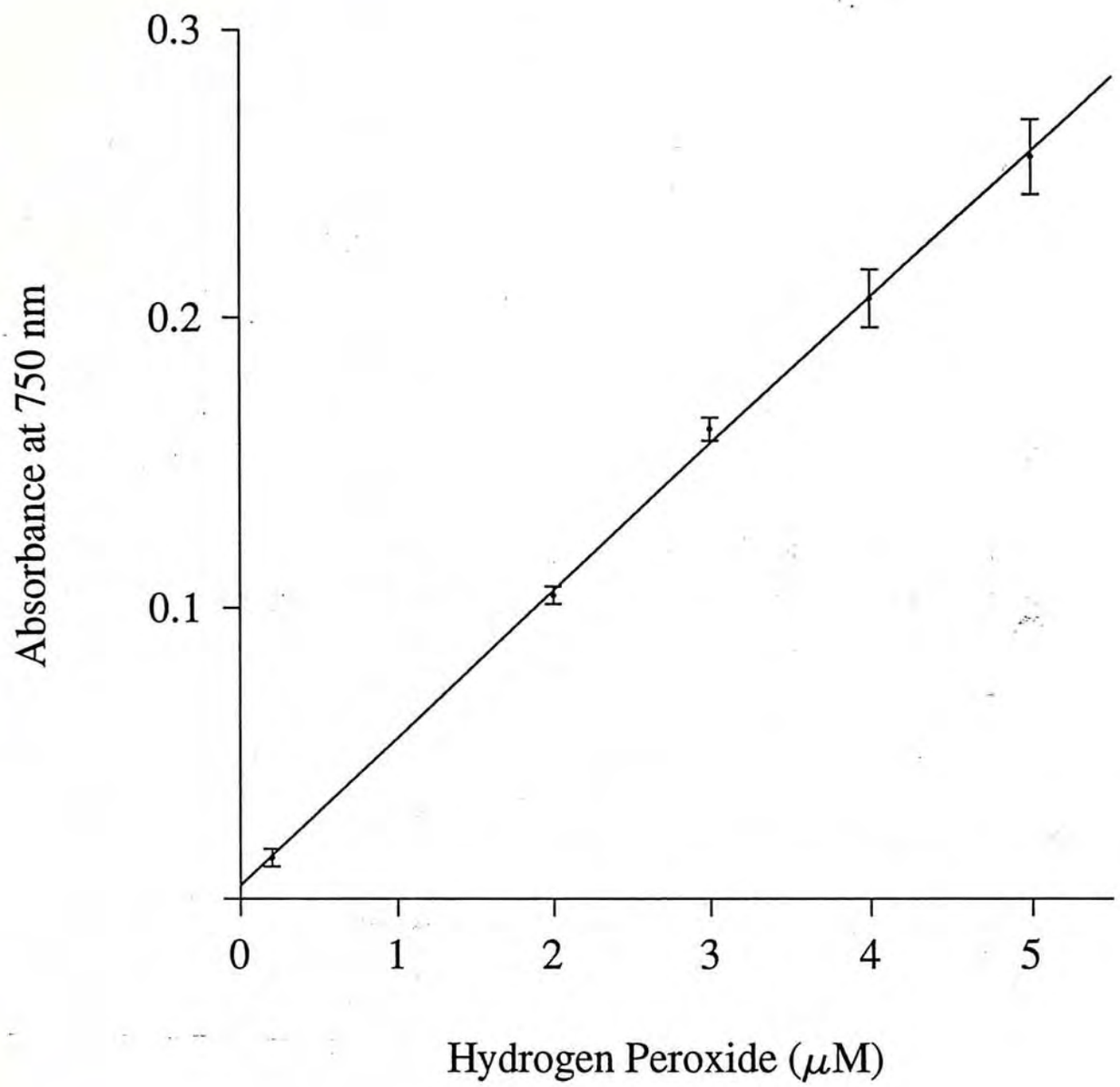


Figure 3.5 Standard curve for hydrogen peroxide assay using hydrogen peroxide as standard

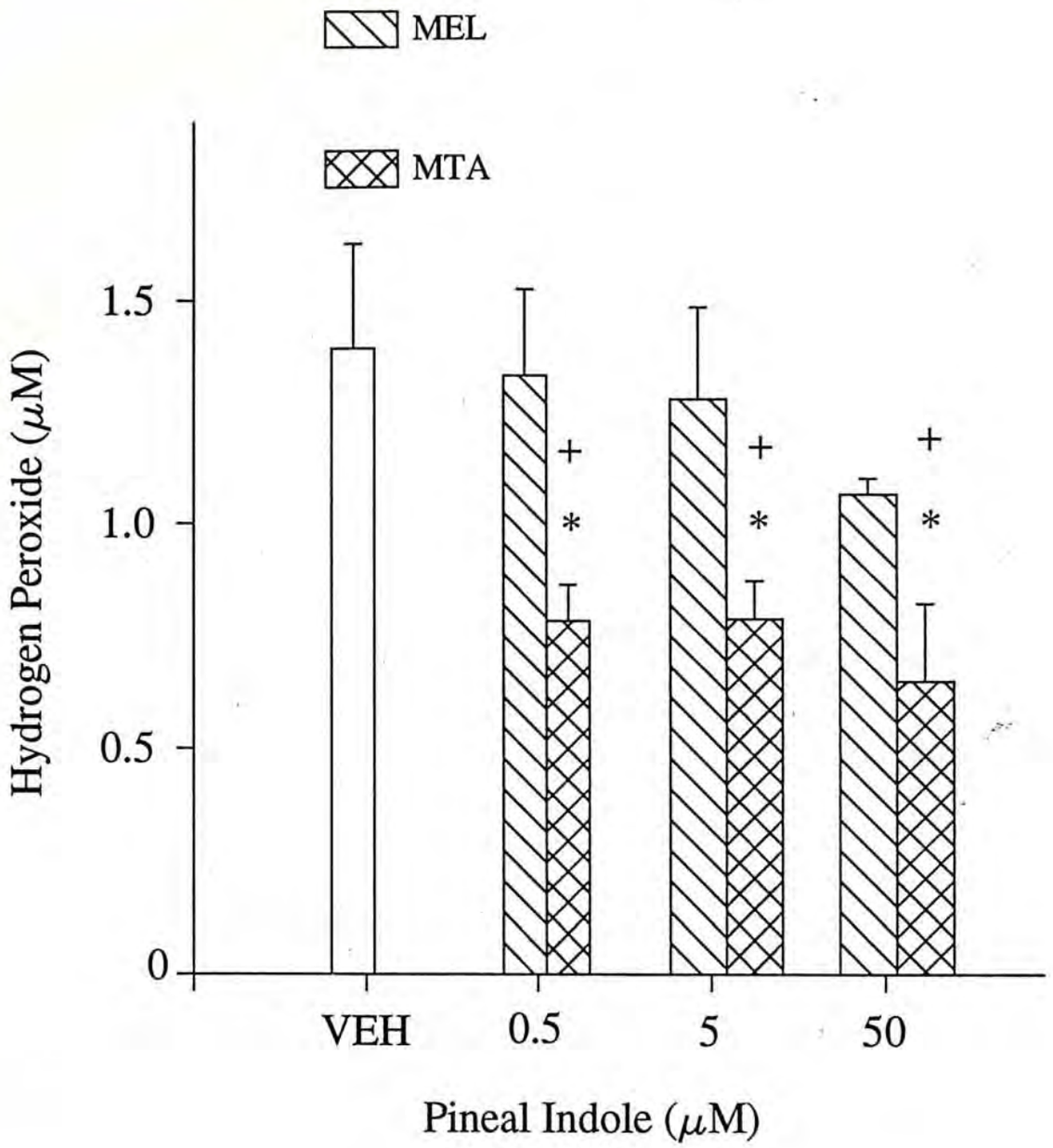


Figure 3.6 In vitro effects of melatonin and methoxytryptamine on hydrogen peroxide production by peritoneal macrophages from C57 mice. Results are expressed as means ± standard deviation (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from corresponding melatonin group. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

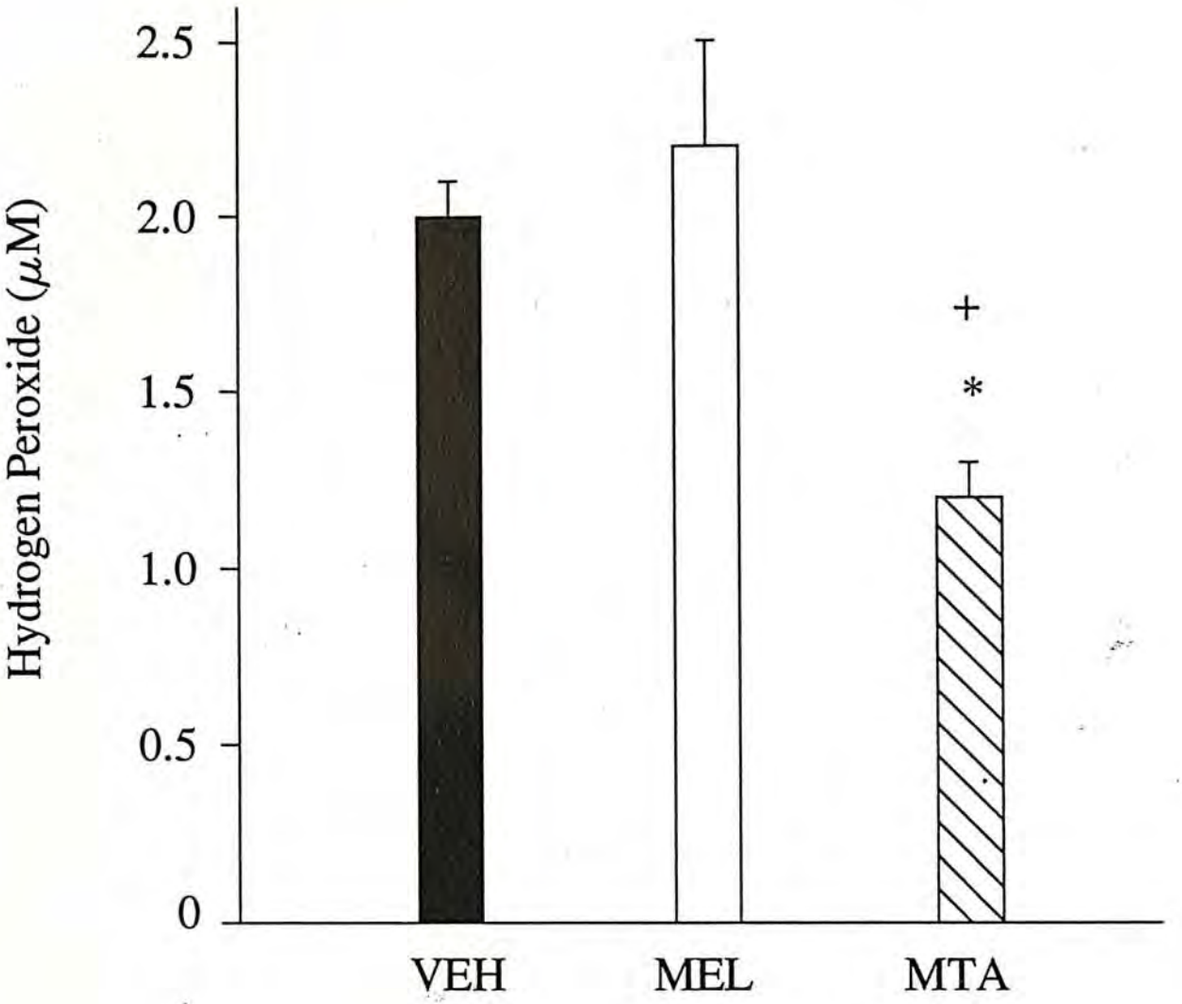


Figure 3.7 In vitro hydrogen peroxide production by peritoneal macrophages from C57 mice (N = 20/group) treated with melatonin or methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks. Results are expressed as means ± standard deviation (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from melatonin group. VEH = control, MEL = melatonin, MTA = methoxy - tryptamine.

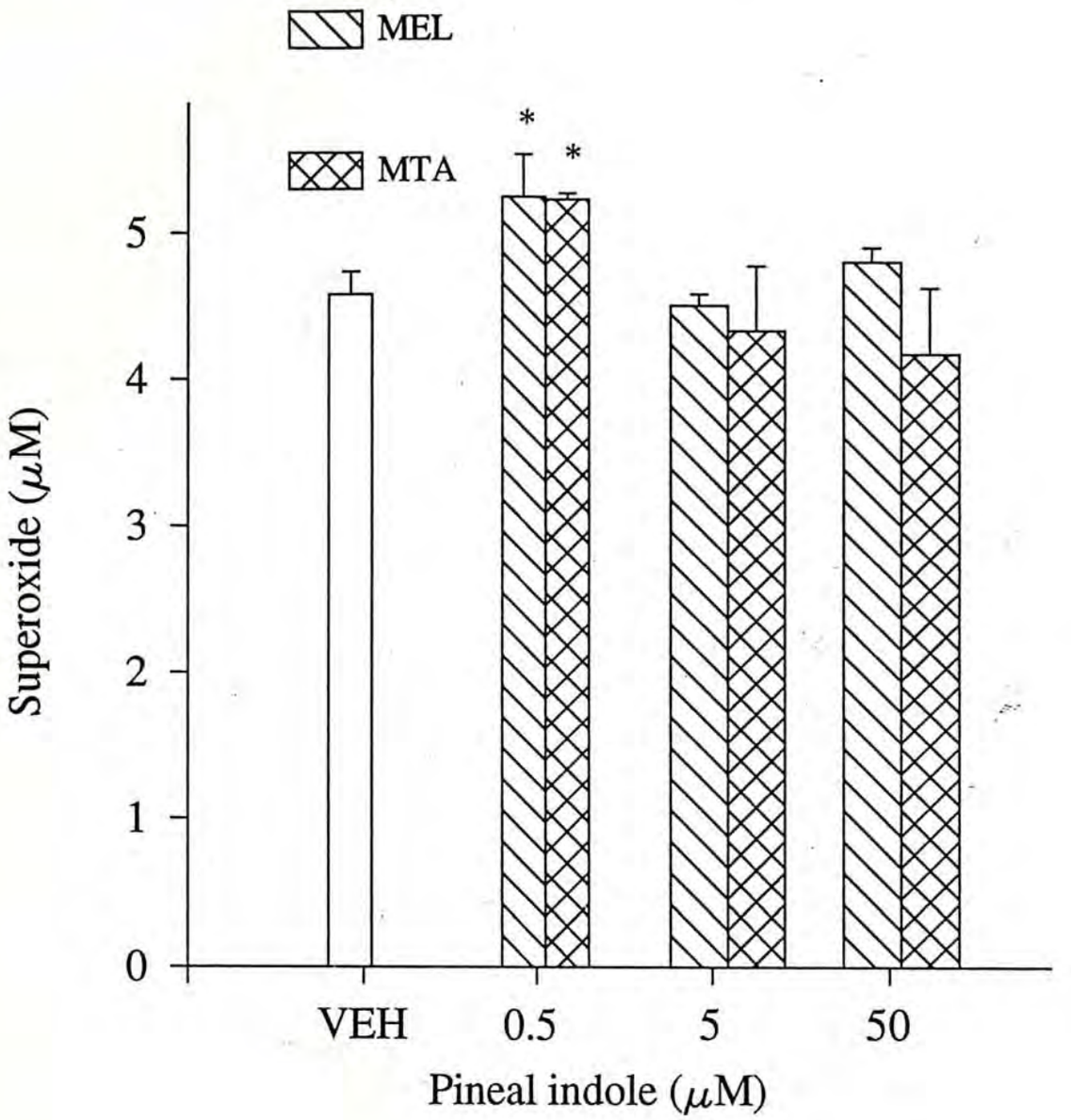


Figure 3.8 In vitro effects of melatonin and methoxytryptamine on superoxide production by peritoneal macrophages from C57 mice. Results are expressed as means ± standard deviation (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

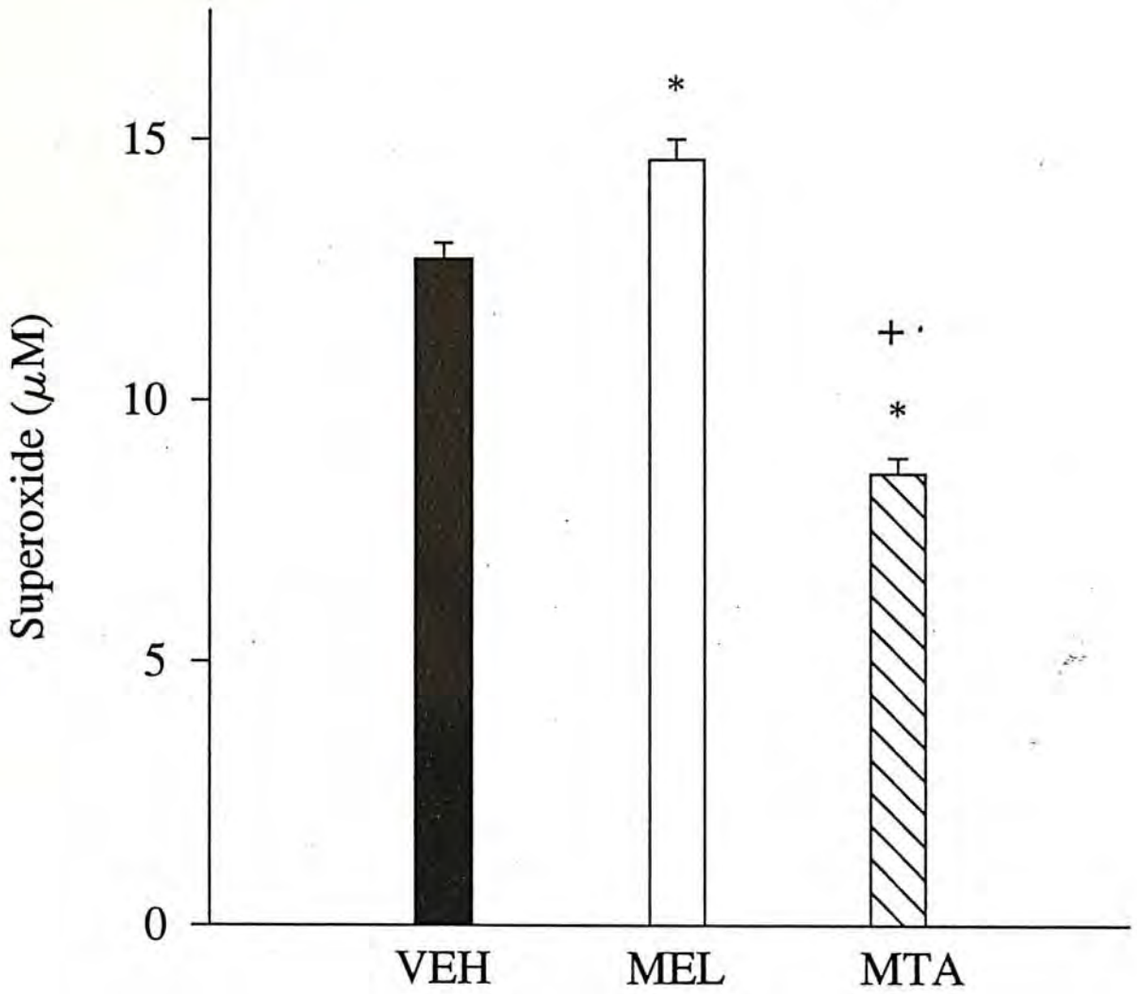


Figure 3.9 In vitro superoxide production by peritoneal macrophages from C57 mice (N = 20/group) treated with melatonin or methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks. Results are expressed as means ± standard deviation (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from melatonin group. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

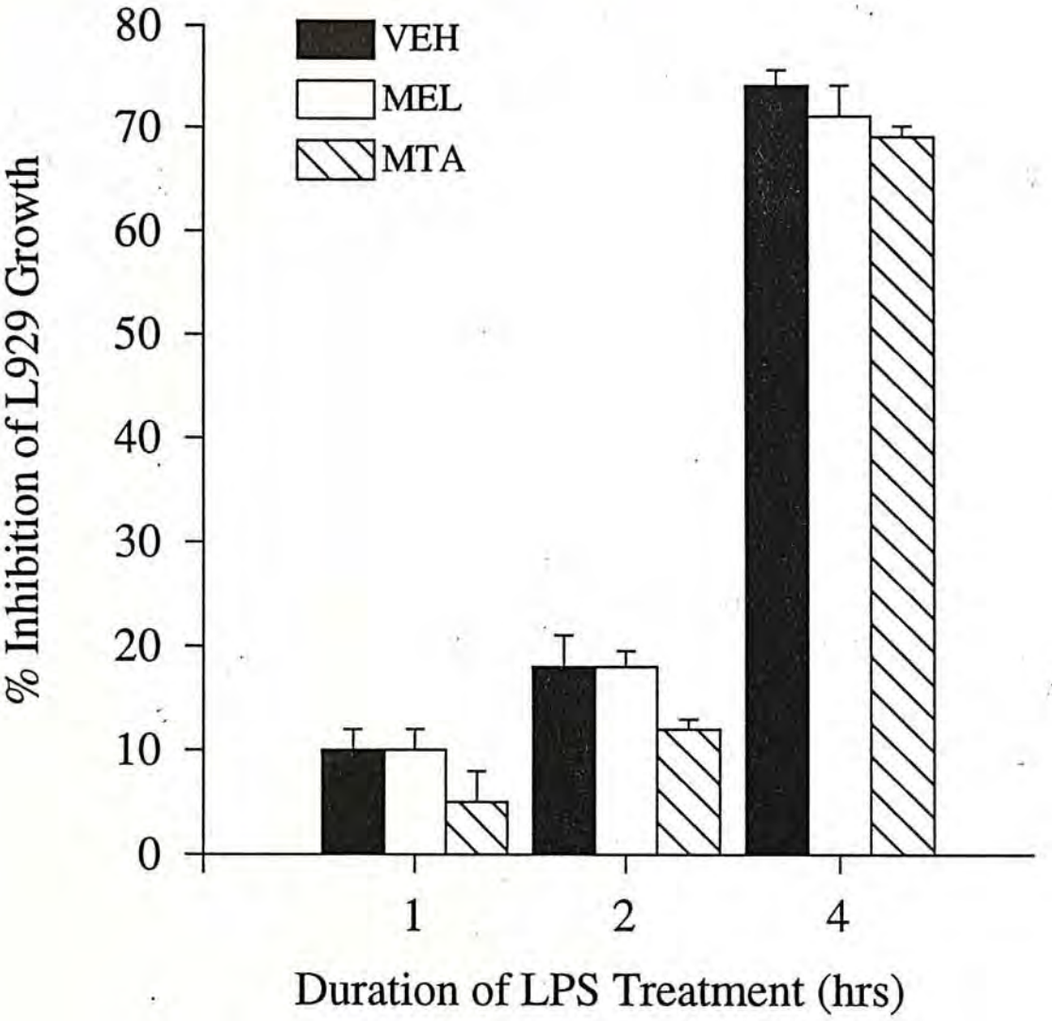


Figure 3.10 In vitro effects of melatonin (50 μM) and methoxytryptamine (50 μM) on production of tumor necrosis factor-α (= inhibtion of L929 growth) by peritoneal macrophages from C57 mice. The tumor necrosis factor-α - containing supernatant was diluted 200 folds for assay. Results are expressed as means ± standard deviation (n = 3). VEH = control, MEL = melatonin, MTA = methoxytryptamine.

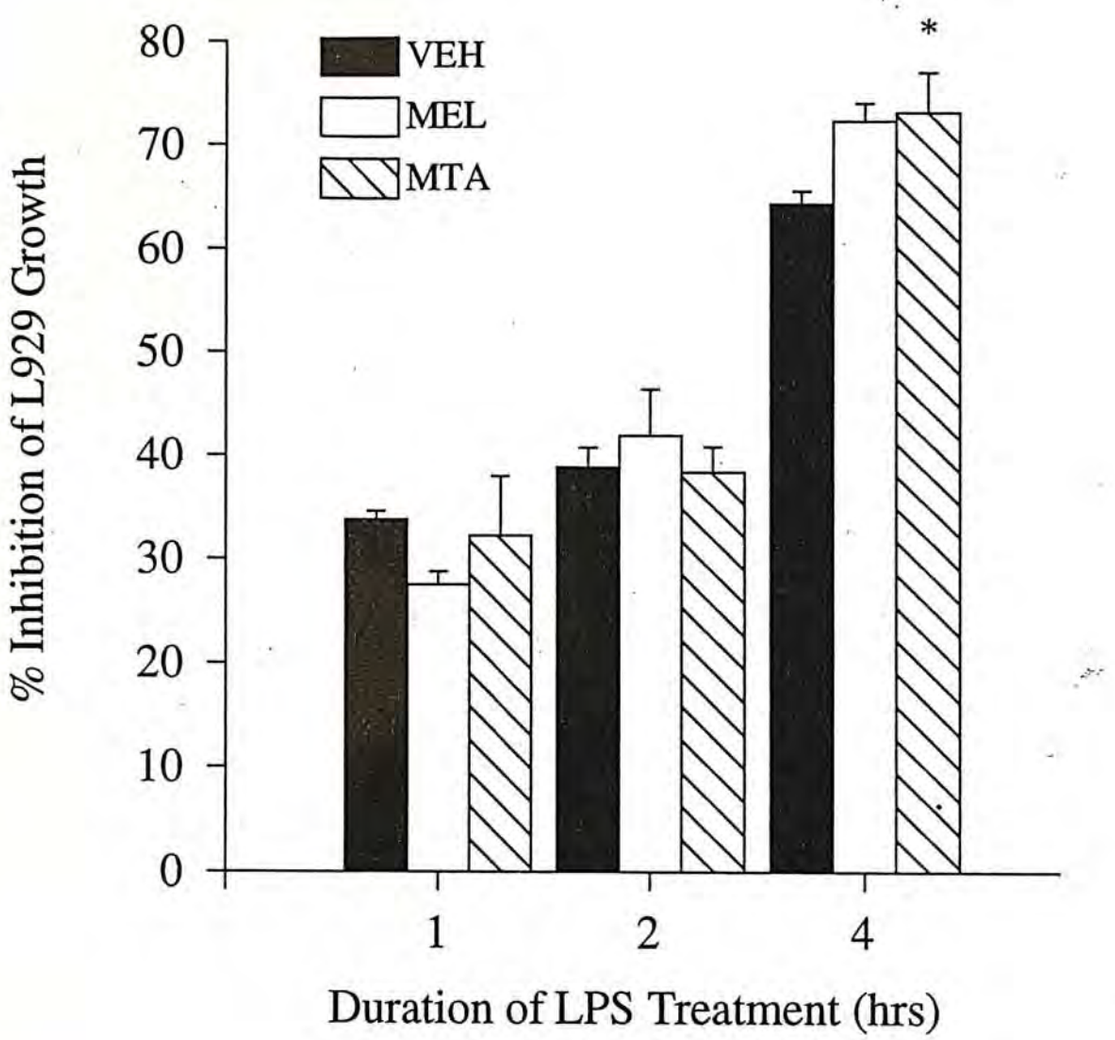


Figure 3.11 In vitro production of tumor necrosis factor-α (= inhibition of L929 growth) by peritoneal macrophages from C57 mice (N = 20/group) which had been treated with melatonin or methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks. The tumor necrosis factor-α containing supernatant was diluted 200 folds before assay. Results are expressed as means ± standard deviation (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. VEH = control, MEL = melatonin, MTA = methoxytryptamine, LPS = lipopolysaccharide.

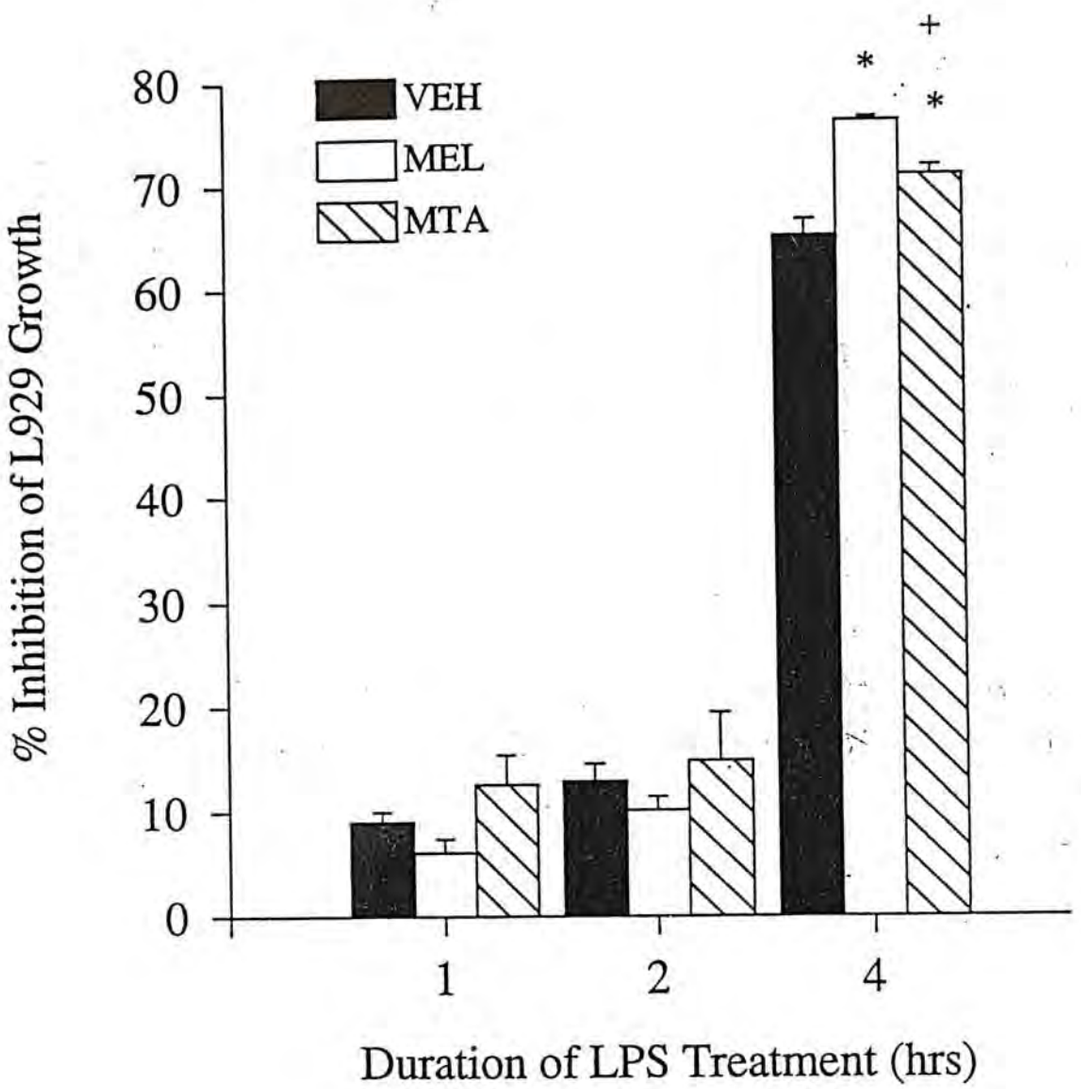


Figure 3.12 In vitro production of tumor necrosis factor-α (= inhibition of L929 growth) by peritoneal macrophages from C57 mice (N = 20/group) which had been treated with melatonin or methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks. The tumor necrosis factor-α containing supernatant was diluted 400 folds before assay. Results are expressed as means ± standard deviation (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from corresponding melatonin group. VEH = control, MEL = melatonin, MTA = methoxytryptamine, LPS = lipopolysaccharide.

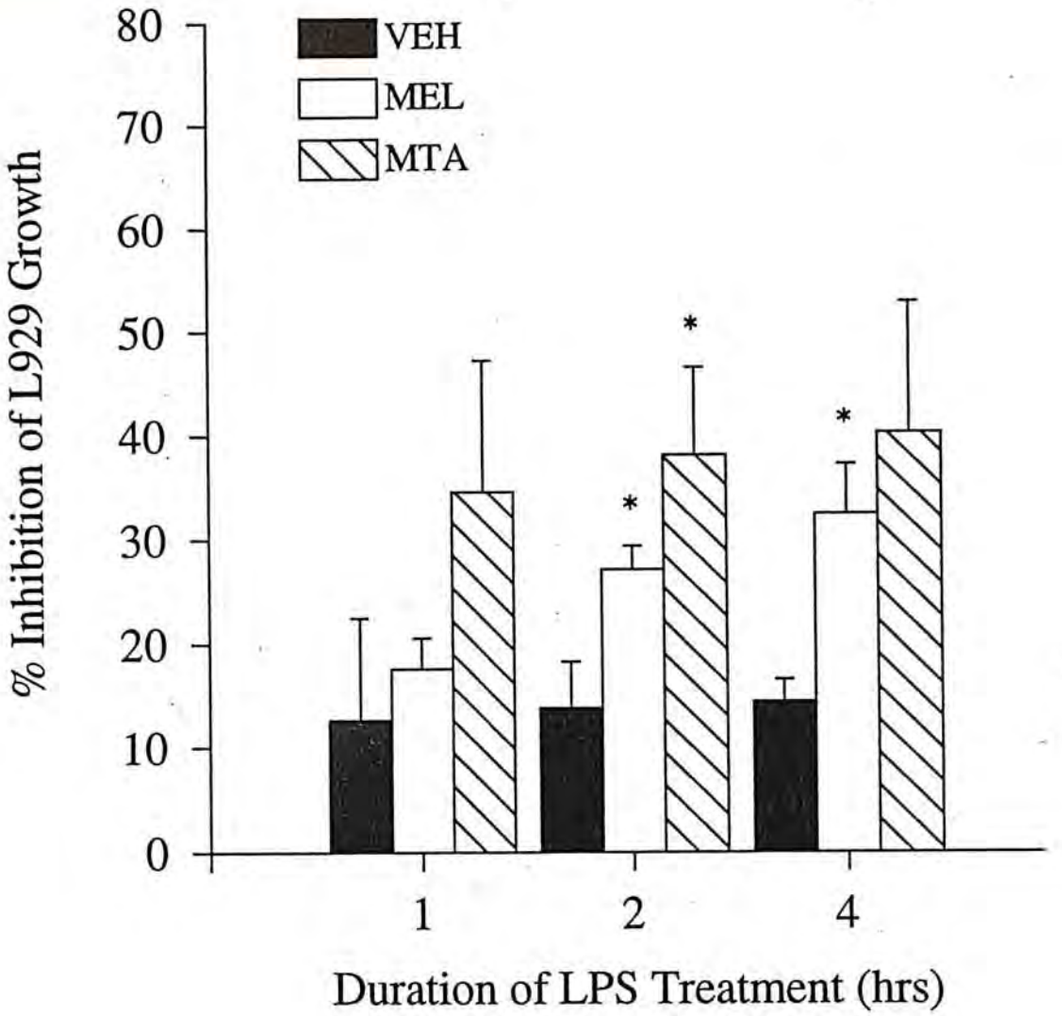
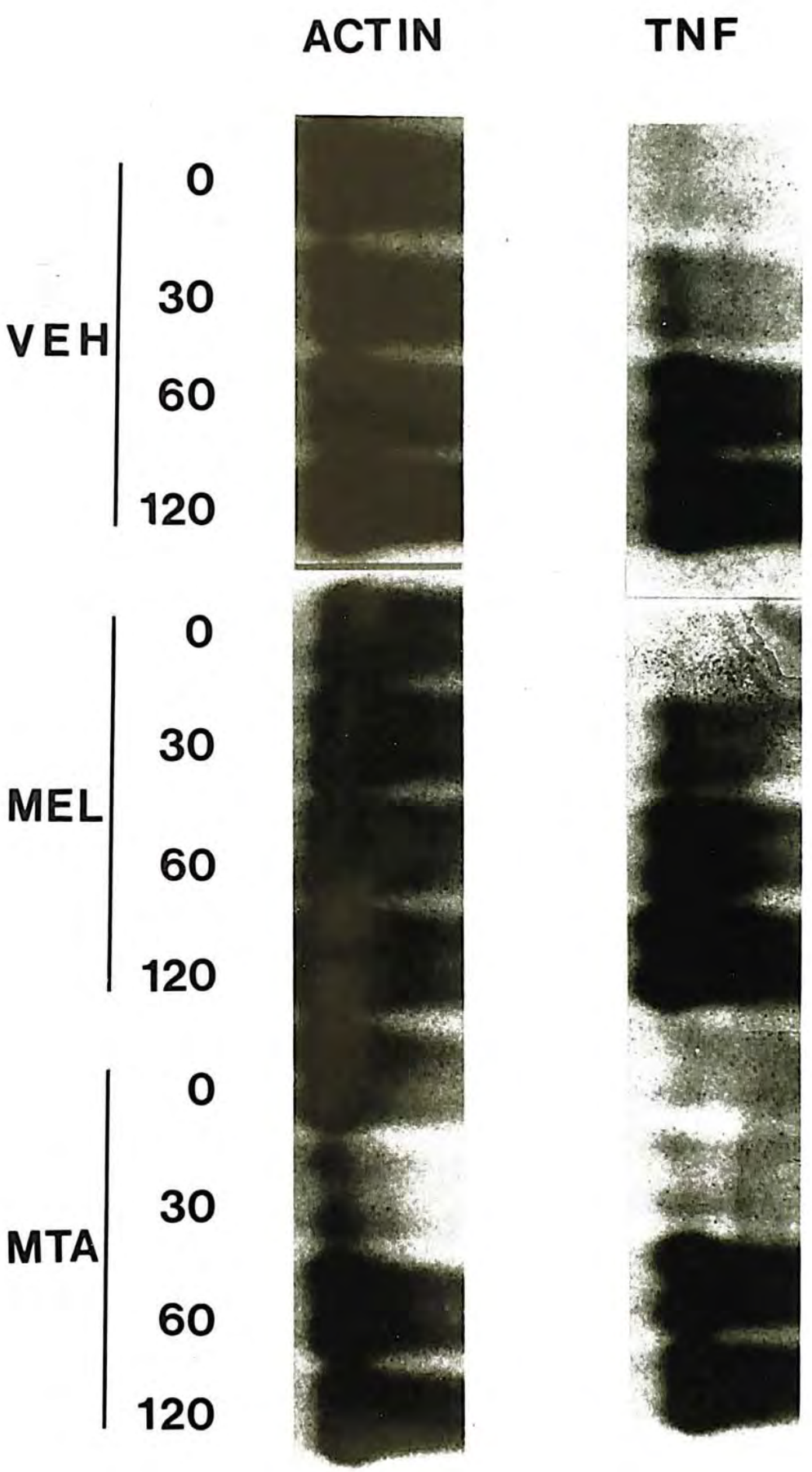


Figure 3.13 In vitro production of tumor necrosis factor-α (= inhibition of L929 growth) by peritoneal macrophages from C57 mice (N = 20/group) which had been treated with melatonin or methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks. The tumor necrosis factor-α containing supernatant was diluted 800 folds before assay. Results are expressed as means ± standard deviation (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. VEH = control, MEL = melatonin, MTA = methoxytryptamine, LPS = lipopolysaccharide.

Figure 3.14 Autoradiographs of actin and TNF-α mRNAs in macrophages from C57 mice (N = 20/group) which had been treated with melatonin or methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks, after treatment with LPS for difference durations. The numbers represent duration of LPS treatment in minutes. VEH = control, MEL = melatonin, MTA = methoxytryptamine, LPS = lipo - polysaccharide, TNF = tumor necrosis factor.


ACTIN
TNF
VEH
0
30
60
120
MEL
0
30
60
120
MTA
0
30
60
120

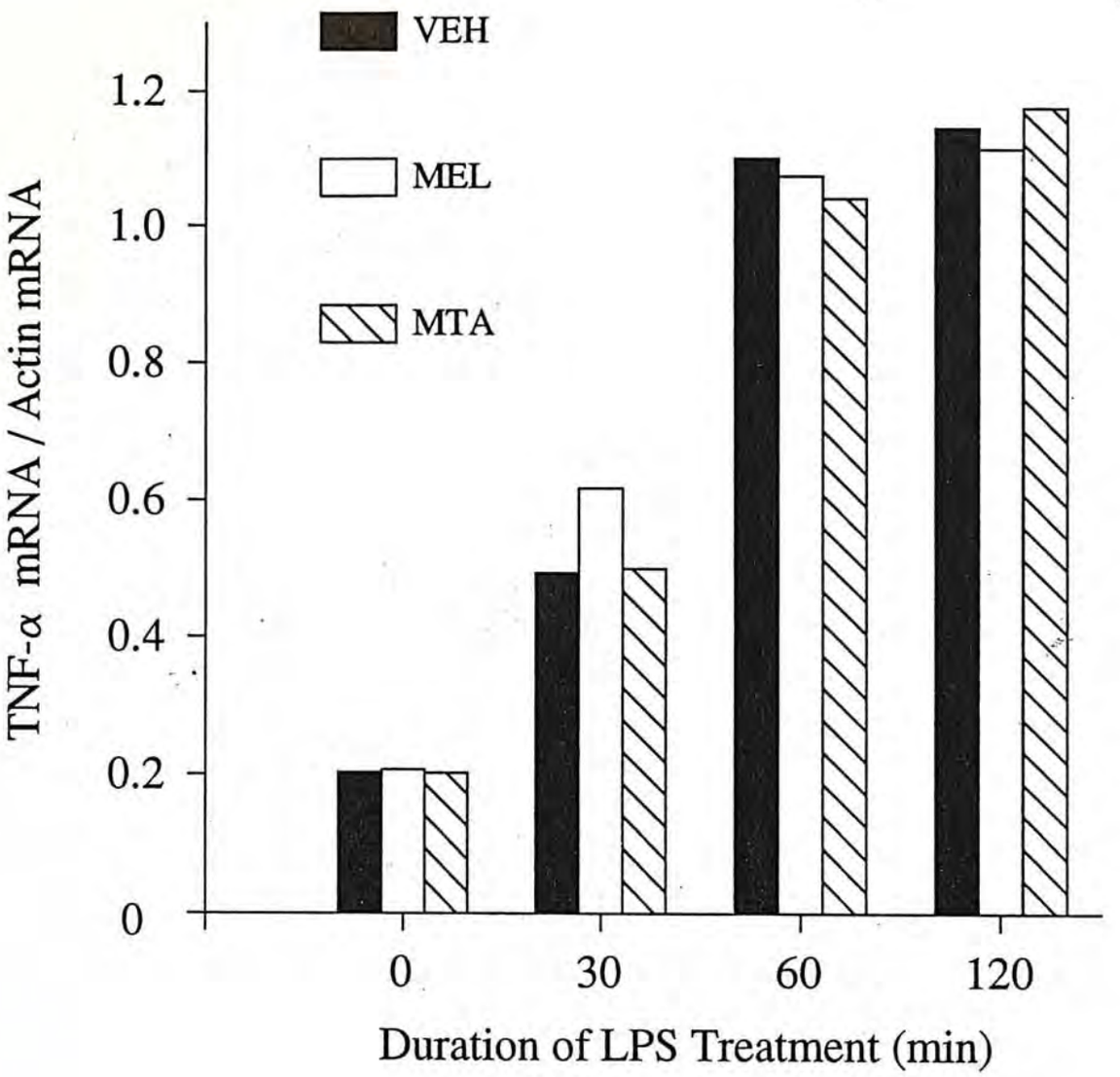


Figure 3.15 Ratio of TNF-α mRNA : actin mRNA in peritoneal macrophages from C57 mice (N = 20/group), which had been treated with melatonin or methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks, after treatment with LPS for difference durations. VEH = control, MEL = melatonin, MTA = methoxytryptamine, LPS = lipopolysaccharide, TNF = tumor necrosis factor.

Chapter 4 Activation of murine splenocytes by melatonin and methoxytryptamine

4.1 Introduction

Pawlikowski (1988) reported that melatonin at concentrations of 10^{-4} - 10^{-8} M exerted no significant influence on ^{3}H-thymidine incorporation by spleen lymphocytes. Arzt (1988) found that only at a concentration in the range of 10^{-3} - 10^{-4} M did melatonin inhibit optimally stimulated proliferation of human peripheral blood T lymphocytes by phytohemagglutinin. However, suboptimally stimulated proliferation of human peripheral blood T lymphocytes by phytohemagglutinin was inhibited by melatonin at a concentration in the range of 10^{-3} - 10^{-7} M.

Maestroni and his colleagues from the Institute for Integrative Biomedical Research in Switzerland have published three papers on role of the pineal gland in immunity. Maestroni (1986) found that melatonin injected into mice was able to antagonize the depression of antibody production by corticosterone in vivo and reverse the suppression of humoral response and autologous mixed lymphocyte reaction by propranolol and p-chlorophenylalanine. Propranolol is a ß-antagonist which antagonizes the action of norepinephrine on the nocturnal rise in pineal hormone production. p-Chlorophenylalanine was able to deplete serotonin in the central nervous system (Sanders 1974). Serotonin is the precursor and limiting substrate of melatonin. Maestroni (1986) found that the opioid receptor blocker, naltrexone, was able to antagonize the enhancing effect of melatonin on primary antibody response

in vivo. He suggested that melatonin enhanced the antibody response via an opiatergic mechanism. Maestroni (1988) found that naltrexone was also able to antagonize the counteracting effect of melatonin on immunosuppression induced by acute stress. Injection of melatonin in the evening was also found to prevent paralysis and death of mice induced by sublethal doses of encephalomyocarditis virus after acute stress (Maestroni 1988). Maestroni (Giorda 1991) published another paper on melatonin - induced enhancement of antibody - dependent cellular cytotoxicity. Administration of melatonin into mice was able to enhance the antibody - dependent cellular cytotoxicity of leukocyte effector cells. The enhancement was not inhibited by the opioid antagonist, naloxone.

Investigations about immunomodulatory effect of pineal indoles concentrated on serotonin and melatonin were few in number and mostly confined to study on lymphocytes. Since melatonin was found to have stimulatory effects on macrophages only in vivo, it was suspected that the indole could also affect the cytokine activity in vivo. Methoxytryptamine, which was not cytotoxic to macrophages, was found to have some stimulatory effect on certain functions of macrophages in vivo. Both indoles were thus studied in this investigation.

4.2 Materials and methods

4.2.1 *Chemicals*

Melatonin (MEL), methoxytryptamine (MTA), lipopolysaccharide (LPS) from

Escherichia coli, Concanavalin A were obtained from Sigma Chemical Co. Stocks of melatonin and methoxytryptamine were prepared by dissolving 50 mg pineal indole in 2 ml 95% alcohol.

4.2.2 *Culture media*

Dulbecco's modified Eagle's medium without phenol red (catalog no. D 2902), Dulbecco's phosphate buffered saline (catalog no. D 5652), Hank's balanced salts (catalog no. H 2387) and RPMI-1640 (catalog no. R 6504) were purchased from Sigma Co. Generally, they were first unpacked and dissolved in 800 ml fresh double-distilled water. They were stirred gently for 3 - 4 hours. 2 g sodium bicarbonate was then added into RPMI, 3.7 g sodium bicarbonate was added into Dulbecco's modified Eagle's medium and 0.35 g sodium bicarbonate was added into Hank's balanced salt solution. 0.1 g $MgCl_2$•6 H_2O and 0.133 g $CaCl_2$•2 H_2O were added into Dulbecco's phosphate-buffered saline. The solution was stirred for a few minutes. The pH of the solution was adjusted to 7.2 while stirring. Then the solution was adjusted to 1 L with fresh double-distilled water. Finally, the medium was sterilized by filtration using sterile 0.22 μm millipore filter. The sterile medium was then transferred to a sterile container for storage. RPMI and Dulbecco's modified Eagle's medium were supplemented with 10 % fetal bovine serum, streptomycin sulfate (100 μg/ml) and penicillin G (100 IU/ml).

4.2.3 *Animals*

Escherichia coli, Concanavalin A were obtained from Sigma Chemical Co. Stocks of melatonin and methoxytryptamine were prepared by dissolving 50 mg pineal indole in 2 ml 95% alcohol.

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Dulbecco's modified Eagle's medium without phenol red (catalog no. D 2902), Dulbecco's phosphate buffered saline (catalog no. D 5652), Hank's balanced salts (catalog no. H 2387) and RPMI-1640 (catalog no. R 6504) were purchased from Sigma Co. Generally, they were first unpacked and dissolved in 800 ml fresh double-distilled water. They were stirred gently for 3 - 4 hours. 2 g sodium bicarbonate was then added into RPMI, 3.7 g sodium bicarbonate was added into Dulbecco's modified Eagle's medium and 0.35 g sodium bicarbonate was added into Hank's balanced salt solution. 0.1 g $MgCl_2 \cdot 6\ H_2O$ and 0.133 g $CaCl_2 \cdot 2\ H_2O$ were added into Dulbecco's phosphate-buffered saline. The solution was stirred for a few minutes. The pH of the solution was adjusted to 7.2 while stirring. Then the solution was adjusted to 1 L with fresh double-distilled water. Finally, the medium was sterilized by filtration using sterile 0.22 μm millipore filter. The sterile medium was then transferred to a sterile container for storage. RPMI and Dulbecco's modified Eagle's medium were supplemented with 10 % fetal bovine serum, streptomycin sulfate (100 μg/ml) and penicillin G (100 IU/ml).

4.2.3 *Animals*

Inbred male C57 BL/6 mice, 8-12 weeks old, were used in this study. One group was provided with 2 ml melatonin solution (25 mg/ml in 95 % ethanol) in 500 ml drinking water, another group with 2 ml methoxytryptamine solution (25 mg/ml in 95 % ethanol) in 500 ml drinking water, while the third group received 2 ml 95 % alcohol in 500 ml drinking water and served as the control. The animals were kept at 21 ± 2 °C under a 14/10 - light/dark cycle so that the animals were physiologically pinealectomized. The animals were maintained on standard rodent chow.

4.2.4 *Preparation of splenocytes for assay*

Animals were killed by cervical dislocation and spleens were aseptically excised from C57 mice and transferred to Hank's Balanced Salt Solution. They were then cut into small pieces and pressed through a 100 - mesh stainless steel sieve aseptically. The cells in the medium were spun down and washed with Hank's Balanced Salt Solution. They were counted and resuspended at 1 X 10^7 cells/ml RPMI medium.

4.2.5 *Preparation of peritoneal macrophages for assay*

3 % thioglycolate solution, which was sterilized and aged for at least 1 month, was injected into control C57 mice to elicit macrophages towards the peritoneal cavity for 3 days. The thioglycolate-elicited peritoneal macrophages were collected by peritoneal lavage with Hanks' balanced salt solution (Liu et al. 1989). The macrophages were resuspended in RPMI 1640 at a concentration of 2×10^6 cells/ml.

4.2.6 *Preparation of crude lymphokines for assay*

15 ml of spleen cells (1 X 10^7 cells/ml) were cultured in a 75 cm^2 culture flask (Sterlin) in RPMI medium which contained 10 μg/ml Concanavalin A (CON A) for 3 hours. The culture medium was decanted and the monolayer of lymphocytes was rinsed carefully with warm phosphate - buffered saline to remove excess CON A. The cells were incubated for an additional 24 hours at 37 °C with 15 ml fresh supplemented RPMI (for the assay of macrophage activating factor) or DMEM without phenol red (for the assay of nitrite). After 24 hours the cell-free supernatant containing crude lymphokines was collected and sterilized by passing through a 0.22 μm millipore filter. It was stored at -70 °C.

4.2.7 *Preparation of scintillation fluid*

0.4 g POPOP and 4 g PPO were dissolved in 700 ml toluene and mixed for 3 hours. 300 ml Triton X-100 was then added to the mixture to bring the volume to 1 L.

4.2.8 *Assay of mitogenic response of splenocytes*

Mitogenic response was determined by incubating 1 X 10^6 splenocytes /0.1 ml/well with an equal volume of different dilutions of CON A or LPS in 96-well flat - bottom microtest plates (Sterlin) at 37 °C in a humidified atmosphere of 5 % CO_2. The cultures were incubated for 48 hours. During the last 6 hours, the cells were pulsed with 0.5 μCi of methyl-^{3}H-thymidine /10 μl /well. Cells were harvested onto

a glass fiber filter paper. Each well of cells was mixed with 1 ml scintillation fluid in a counting vial. The radioactivity was measured using a Beckman scintillation counter. The mitogenic response was expressed as the stimulation index, which was the ratio of uptake of ^{3}H-thymidine by splenocytes treated with CON A to uptake of ^{3}H-thymidine by control splenocytes cultured in medium. The lack of an adverse effect of pineal indoles on viability of splenocytes was established by the trypan blue exclusion test.

4.2.9 *Assay of γ-interferon production by splenocytes*

Crude lymphokines were used to test for the concentration of γ-interferon. Using an InterTest-γ™ Mouse IFN-γ ELISA Test Kit from Genzyme Corporation (Genzyme catalog no. 1557-00).

4.2.9.1 *Coating plate with capture reagent (first antibody)*

38 µl of monoclonal anti-mIFN-γ (Genzyme catalog no. 1547-01) was diluted into 11 ml coating buffer (Genzyme catalog no. 1168-00). Diluted monoclonal antibody (100 µl) was added to each well of the 96 - well plate. The plate was then incubated overnight at 4 °C.

4.2.9.2 *Preparation of assay solutions*

The washing buffer was prepared by reconstituting the PBS powder (Genzyme

catalog no. 1175-00) in 2 liters of distilled water containing 1 ml of surfactant (Genzyme catalog no. 1167-00). Blocking/dilution buffer was prepared by adding 15 ml of concentrated blocker/diluent (Genzyme catalog no. 1463-00) to 60 ml Washing Buffer. 2 N sulfuric acid was prepared by diluting concentrated sulfuric acid 5 - fold with distilled water.

4.2.9.3 *Blocking the plate*

The coating mixture in the plate was decanted. The plate was washed with washing buffer and dried. 200 µl blocking/dilution buffer was added into each well. The plate was incubated at 37 °C for 30 minutes.

4.2.9.4 *Incubation of Standard and Sample*

Different dilutions of standard (Genzyme catalog no 1549-00) were prepared. The blocking buffer in the plate was decanted and the plate was dried. The standard and the samples were added into the plate. The plate was then incubated at room temperature for 2 hours.

4.2.9.5 *Addition of Second Antibody*

The solution in the plate was decanted and the plate was washed with washing buffer four times. 440 µl of goat anti-IFN-γ (Genzyme catalog no. 1548-00) was diluted into 10.5 ml blocking/dilution buffer. 100 µl of diluted secondary antibody was

added into each well of the plate. The plate was incubated at room temperature for 2 hours.

4.2.9.6 *Addition of Third Antibody*

The solution in the plate was decanted. The plate was washed with washing buffer four times and dried. 11 µl of peroxidase - conjugated donkey anti - goat (Genzyme catalog no. 1465-00) antiserum was diluted into 11 ml blocking/dilution buffer. 100 µl of diluted third antibody was added into each well of the plates. The plate was then incubated at room temperature for one hour.

4.2.9.7 *Addition of Substrate and stopping color development*

50 µl of peroxide solution was diluted in 11 ml substrate buffer (Genzyme catalog no. 1171-00). 2 OPD (O-phenylenediamine dihydrochloride) tablets (Genzyme catalog no. 1508-01) were added into the substrate buffer/peroxide solution and mixed gently to dissolve. The resulting solution was the substrate reagent. The solution in the plate was decanted. The plate was washed with washing buffer four times and dried. 100 µl of substrate reagent was added into each well of the plate. The reaction was left to develop for 4 - 6 minutes at room temperature. 100 µl of 2 N sulfuric acid was added into each well of the plate to stop color development. Absorbance at 490 nm was then read to measure the level of IFN-γ in the tested crude lymphokines.

4.2.10 *Assay of interleukin - 2 production by splenocytes*

It was determined according to the method described by Gillis (1978). Different dilutions of interleukin - 2 standard from Genzyme (code MIL-2) were used to stimulate the growth of CTLL 2 cells (mouse T cells from ATCC, catalog no. TIB 214). 2 X 10^4 CTLL 2 cells/50 µl/well were seeded into a 96 - well plate. 50 µl of different dilutions of the standard or samples was added into the plate. The plate was incubated at 37 °C in a humidified atmosphere of 5 % CO_2. The cultures were incubated for 48 hours. During the last 6 hours, the cells were pulsed with 0.5 µCi of methyl-^{3}H-thymidine (Amersham)/10 µl/well. Afterwards the cells were harvested onto a glass fiber filter paper. Each well of cells was mixed with 1 ml scintillation fluid in a counting vial. The radioactivity was measured using a Beckman scintillation counter and the concentration of interleukin - 2 was expressed as U/ml.

4.2.11 *Assay of nitrite production by macrophages after stimulation by lymphokines*

Thioglycolate - elicited peritoneal macrophages were collected and washed with Hanks' balanced salt solution. They were resuspended at a concentration of 2 X 10^6 cells/ml supplemented DMEM without phenol red. The cells (2 X 10^5/0.1 ml/well) were allowed to adhere onto the surface of the 96 - well culture plate for 2 hours before they were challenged with crude lymphokines (containing 10 ng LPS /ml) from different groups of mice for 1 day. The supernatant was then allowed to react with half its volume of Griess reagent (1 % sulfanilamide in 5 % H_3PO_4 - 0.1 %

naphthalene-ethylenediamine dihydrochloride) for 15 minutes and the level of nitrite present in the culture medium was measured by an ELISA reader at 540 nm. The concentration of nitrite was estimated using $NaNO_2$ as the standard and expressed as μM.

4.2.12 *Assay of production of macrophage activating factor (MAF) by splenocytes*

Tumoricidal activity of MAF-activated macrophages was assayed with a modification of the method of Taramelli (1980). Serial dilutions (1:1) of a suspension (2 X 10^6 cells/ml) of thioglycolate - elicited peritoneal macrophages were prepared. Different dilutions of peritoneal macrophages was seeded into a 96 - well plates. Crude lymphokines containing 10 ng LPS /ml in 1 : 1 dilution were used to stimulate the peritoneal macrophages at 37 °C for 24 hours. The peritoneal macrophages were then washed with warm phosphate - buffered saline two times. 100 μl of murine mastocytoma P815 cells at a concentration of 5 X 10^4 cells/ml was then added into each well of the plate and incubated at 37 °C for 48 hours. During the last 6 hours, the cells were pulsed with 0.5 μCi of methyl-^{3}H-thymidine (Amersham) /10 μl/well. The cells were then harvested on a glass fiber filter paper. Each well of cells was mixed with 1 ml scintillation fluid in a counting vial. The radioactivity was measured using a Beckman scintillation counter.

4.2.13 *Statistics*

The data were analyzed by one - way analysis of variance using Scheffe's multiple

range test. The levels of significance were taken at $p < 0.05$.

4.3 Results

4.3.1 *Mitogenic response of mouse splenocytes to CON A. The mice had been treated with melatonin and methoxytryptamine in the drinking water for 2 weeks.*

Splenocytes from mice treated with vehicle), melatonin, or methoxytryptamine in the drinking water were respectively cultured with different concentrations of CON A for 48 hours. Their ^{3}H-thymidine uptakes were then used to calculate the stimulation index. The stimulation index is the ratio of ^{3}H-thymidine uptake of splenocytes cultured with CON A to ^{3}H-thymidine uptake of splenocytes cultured in medium. The result is shown in Figure 4.1. The mitogenic response to CON A was the greatest in the melatonin - treated group (about 25 % higher than the control group when 10 μg CON A /ml was used). The mitogenic response of the methoxytryptamine - treated group to CON A was lower than the control group (about 20 % smaller than the control group when 10 μg CON A /ml was used). The differences were distinct when 10 μg CON A /ml was used. Smaller differences found at higher concentrations of CON A may have been due to overgrowth of the splenocytes.

4.3.2 *Mitogenic response of mouse splenocytes to lipopolysaccharide (LPS). The mice had been treated with melatonin and methoxytryptamine in the*

drinking water for 2 weeks

Splenocytes from mice treated with the vehicle, melatonin, and methoxytryptamine respectively in the drinking water were cultured with different concentrations of LPS for 48 hours. Their ^{3}H-thymidine uptakes were then used to calculate the stimulation index. The stimulation index is the ratio of ^{3}H-thymidine uptake of splenocytes cultured with LPS to ^{3}H-thymidine uptake of splenocytes cultured in medium. The result is shown in Figure 4.2. The mitogenic response to LPS was the greatest in the melatonin - treated group (about 100 % higher than the control group when 4 μg LPS /ml was used). The mitogenic response of the methoxytryptamine - treated group to LPS was about 70 % higher than the control group when 4 μg LPS /ml was used. The trends were similar at other concentrations of LPS although the differences were smaller.

4.3.3 *In vitro production of γ-interferon by mouse splenocytes from mice which had been treated with melatonin and methoxytryptamine in the drinking water for 2 weeks*

Splenocytes from mice treated with vehicle, melatonin, and methoxytryptamine respectively in the drinking water were pre - treated with 10 μg CON A /ml for 3 hours and cultured for 24 hours. The supernatant was used to test for the activity of γ-interferon. The standard curve of γ-interferon was constructed according to the method described in the instructions accompanying the InterTest-γ™ Mouse IFN-γ ELISA Test Kit and is shown in Figure 4.3. 8000 pg γ-interferon standard /ml was

equivalent to about 36 units/ml. The result is shown in Figure 4.4. Splenocytes from melatonin - treated mice produced about 70 % more γ-interferon than splenocytes from control mice significantly. Splenocytes from methoxytryptamine - treated mice produced about 50 % more γ-interferon than splenocytes from control mice significantly.

4.3.4 *In vitro production of interleukin-2 by splenocytes from mice which had been treated with melatonin and methoxytryptamine in the drinking water for 2 weeks*

Splenocytes from mice treated with vehicle, melatonin, and methoxytryptamine respectively in the drinking water were pre - treated with 10 μg CON A /ml for 3 hours and cultured for 24 hours. The supernatant was used to test the activity of interleukin-2. The standard curve of interleukin-2 was constructed by using different dilutions of interleukin-2 from Genzyme to stimulate the growth of the CTLL 2 cells (Figure 4.5). The linear portion of the curve was then used for calculation (Figure 4.6). The result is shown in Figure 4.7. Splenocytes from melatonin - treated mice produced about 3 times as much interleukin-2 as splenocytes from control mice. Splenocytes from methoxytryptamine - treated mice produced about twice as much interleukin-2 as splenocytes from control mice.

4.3.5 *In vitro nitrite production by peritoneal macrophages of mice after stimulation by crude lymphokines from mice which had been treated with melatonin and methoxytryptamine in the drinking water for 2 weeks*

Splenocytes from mice treated with control, melatonin, and methoxytryptamine respectively in the drinking water were pre - treated with 10 μg CON A /ml for 3 hours and cultured for 24 hours. The supernatant was used to stimulate production of nitrite by peritoneal macrophages. The standard curve of nitrite was constructed by using sodium nitrite as standard (Figure 4.8). The result is shown in Figure 4.9 - 4.14.

4.3.5.1 *In vitro nitrite production by peritoneal macrophages of control mice after stimulation by crude lymphokines from mice which had been treated with melatonin and methoxytryptamine in the drinking water for 2 weeks (Figure 4.9)*

Crude lymphokines from melatonin - treated mice were able to stimulate peritoneal macrophages from control mice to produce an about 40 % increase in nitrite over that stimulated by crude lymphokines from control mice. Crude lymphokines from methoxytryptamine - treated mice were not able to stimulate peritoneal macrophages from control mice to produce an increase in nitrite production over that stimulated by crude lymphokines from control mice.

4.3.5.2 *In vitro nitrite production by peritoneal macrophages of melatonin - treated mice after stimulation by crude lymphokines from mice which had been treated with melatonin and methoxytryptamine in the drinking water for 2 weeks (Figure 4.10)*

Crude lymphokines from melatonin - treated mice were able to stimulate peritoneal macrophages from melatonin - treated mice to produce an about 8 - fold increase in nitrite production over that stimulated by crude lymphokines from control mice. Crude lymphokines from methoxytryptamine - treated mice were able to stimulate peritoneal macrophages from melatonin - treated mice to produce an approximately 4 - fold increase in nitrite production over that stimulated by crude lymphokines from control mice.

4.3.5.3 *In vitro nitrite production by peritoneal macrophages of methoxytryptamine - treated mice after stimulation by crude lymphokines from mice which had been treated with melatonin and methoxytryptamine in the drinking water for 2 weeks (Figure 4.11)*

Crude lymphokines from melatonin - treated mice were able to stimulate peritoneal macrophages from methoxytryptamine - treated mice to produce an approximately 5 - fold increase in nitrite production over that stimulated by crude lymphokines from control mice significantly. Crude lymphokines from methoxytryptamine - treated mice were able to stimulate the peritoneal macrophages from methoxytryptamine - treated mice to produce an approximately 3 - fold increase in nitrite production over that stimulated by crude lymphokines from control mice.

4.3.5.4 *In vitro nitrite production by peritoneal macrophages from mice which had been treated with melatonin and methoxytryptamine in the drinking water for 2 weeks. The macrophages had been subjected to in vitro stimulation*

by crude lymphokines from control mice (Figure 4.12).

The response of peritoneal macrophages from methoxytryptamine - treated mice to crude lymphokines from control mice in nitrite production was approximately 40 % higher than that produced by peritoneal macrophages from control mice. The response of peritoneal macrophages from melatonin - treated mice to crude lymphokines from control mice was not significantly different when compared to that produced by peritoneal macrophages from control mice.

4.3.5.5 *In vitro nitrite production by peritoneal macrophages from mice which had been treated with melatonin and methoxytryptamine in the drinking water for 2 weeks. The macrophages had been subjected to in vitro stimulation by crude lymphokines from melatonin - treated mice (Figure 4.13).*

The response of peritoneal macrophages from melatonin - treated mice to crude lymphokine from melatonin - treated mice in nitrite production was about 30 % higher than that produced by peritoneal macrophages from control mice. The response of peritoneal macrophages from methoxytryptamine - treated mice to crude lymphokines from melatonin - treated mice was approximately 70 % higher than that produced by peritoneal macrophages from control mice.

4.3.5.6 *In vitro nitrite production by peritoneal macrophages from mice treated with melatonin and methoxytryptamine in the drinking water for 2 weeks. The macrophages had been subjected to in vitro stimulation by crude*

lymphokines from methoxytryptamine - treated mice (Figure 4.14).

The response of peritoneal macrophages from melatonin - treated mice to crude lymphokines from methoxytryptamine - treated mice in nitrite production was about 50 % higher than that produced by peritoneal macrophages from control mice. The response of peritoneal macrophages from methoxytryptamine - treated mice to crude lymphokines from methoxytryptamine - treated mice was about 70 % higher than that produced by peritoneal macrophages from control mice.

4.3.6 *Production of macrophage activating factor by splenocytes after treatment with melatonin and methoxytryptamine*

The assay did not directly monitor the amount of macrophage activating factor. It was reflected by the ability of macrophages to kill murine mastocytoma P815 cells after stimulation. Splenocytes from mice treated with melatonin and methoxytryptamine respectively in the drinking water were pre - treated with 10 μg CON A /ml for 3 hours and cultured for 24 hours. 1/2 Dilution of the supernatant containing crude lymphokines was then used to stimulate the peritoneal macrophages. The inhibition of the growth of murine mastocytoma P815 cells was calculated from ^{3}H-thymidine uptake of murine mastocytoma P815 cells. The result is shown in Figure 4.15 - 4.20.

4.3.6.1 *<u>In vitro</u> killing of murine mastocytoma P815 cells by peritoneal macrophages of mice which had been treated with melatonin or*

methoxytryptamine in the drinking water for 2 weeks. The macrophages had been subjected to in vitro stimulation by crude lymphokines from control mice (Figure 4.15).

Crude lymphokines from control mice were able to activate peritoneal macrophages from control mice to produce about 35 % inhibition of the growth of murine mastocytoma P815 cells. There was no significant difference in the response of peritoneal macrophages from different groups of mice to activation by crude lymphokines from control mice.

4.3.6.2 *In vitro killing of murine mastocytoma P815 cells by peritoneal macrophages of mice which had been treated with melatonin or methoxytryptamine in the drinking water for 2 weeks. The macrophages had been subjected to in vitro stimulation by crude lymphokines from melatonin - treated mice (Figure 4.16).*

Crude lymphokines from melatonin - treated mice were able to activate peritoneal macrophages from melatonin - treated mice to produce about 90 % inhibition of the growth of murine mastocytoma P815 cells, which was about 35 % higher than that achieved by macrophages from control mice when the ratio of the number of macrophages to the number of murine mastocytoma P815 cells was 40 to 1. Crude lymphokines from melatonin - treated mice were able to activate peritoneal macrophages from methoxytryptamine - treated mice to produce 98 % inhibition of the growth of murine mastocytoma P815 cells, which was about 50 % higher than

that achieved by macrophages from control mice when the ratio of the number of macrophages to the number of murine mastocytoma P815 cells was 40 to 1.

4.3.6.3 *In vitro killing of murine mastocytoma P815 cells by peritoneal macrophages of mice which had been treated with melatonin or methoxytryptamine in the drinking water for 2 weeks. The macrophages had been subjected to in vitro stimulation by crude lymphokines from methoxytryptamine - treated mice (Figure 4.17).*

Crude lymphokines from methoxytryptamine - treated mice were able to activate peritoneal macrophages from control mice to produce about 35 % inhibition of the growth of murine mastocytoma P815 cells. There was no significant difference in the response of peritoneal macrophages from different groups of mice to activation by crude lymphokines of mice from methoxytryptamine - treated mice.

4.3.6.4 *In vitro killing of murine mastocytoma P815 cells by peritoneal macrophages of control mice after in vitro stimulation by crude lymphokines from melatonin - treated or methoxytryptamine - treated mice (Figure 4.18)*

Crude lymphokines from melatonin - treated mice were able to produce about 70 % inhibition of the growth of murine mastocytoma P815 cells by macrophages from control mice, which was about 1 - fold higher than that stimulated by crude lymphokines from control mice when the ratio of the number of macrophages to murine mastocytoma P815 cells was 40 to 1. The activity of macrophage activating

factor in the crude lymphokines from methoxytryptamine - treated mice was not significantly different from that in the crude lymphokines from control mice.

4.3.6.5 *In vitro killing of murine mastocytoma P815 cells by peritoneal macrophages from melatonin - treated mice after in vitro stimulation by crude lymphokines from melatonin - treated or methoxytryptamine - treated mice (Figure 4.19)*

When the ratio of the number of macrophages to the number of murine mastocytoma P815 cells was 40 to 1, crude lymphokines from melatonin - treated mice were able to produce about 90 % inhibition of the growth of murine mastocytoma P815 cells by macrophages from melatonin - treated mice, which was about 3.5 - fold higher than that stimulated by crude lymphokines from control mice. When the ratio of the number of macrophages to the number of murine mastocytoma P815 cells was 20 to 1, crude lymphokines from melatonin - treated mice were able to stimulate about 40 % inhibition of the growth of murine mastocytoma P815 cells by macrophages from melatonin - treated mice, which was about 1 - fold higher than that stimulated by crude lymphokines from control mice. The activity of macrophage activating factor in the crude lymphokines from methoxytryptamine - treated mice was not significantly different when compared to the crude lymphokine from control mice.

4.3.6.6 *In vitro killing of murine mastocytoma P815 cells by peritoneal macrophages from methoxytryptamine - treated mice after in vitro stimulation by crude lymphokines from melatonin - treated or*

methoxytryptamine - treated mice (Figure 4.20)

When the ratio of the number of macrophages to the number of murine mastocytoma P815 cells was 40 to 1, crude lymphokines from melatonin - treated mice were able to produce about 98 % inhibition of the growth of murine mastocytoma P815 cells by macrophages from methoxytryptamine - treated mice, which was about 2.3 - fold higher than that stimulated by crude lymphokine from control mice. When the ratio of the number of macrophages to murine mastocytoma P815 cells was 20 to 1, crude lymphokines from melatonin - treated mice were able to produce about 40 % inhibition of the growth of murine mastocytoma P815 cells by macrophages from methoxytryptamine - treated mice, which was about 1 - fold higher than that stimulated by crude lymphokines from control mice. The activity of macrophage activating factor in the crude lymphokines from methoxytryptamine - treated mice was not significantly different when compared to crude lymphokines from control mice, when the ratio of the number of macrophages to the number of murine mastocytoma P815 cells was 20 to 1. The activity of macrophage activating factor in the crude lymphokines from methoxytryptamine - treated mice was shown to be about 50 % lower than that of control mice when the ratio of the number of macrophages to the number of murine mastocytoma P815 cells was 40 to 1.

4.4 Discussion

Several conclusions about melatonin can be drawn from the results. Firstly, splenocytes from melatonin - treated mice exhibited greater mitogenic responses to

CON A (25 %) and LPS (100 %) than splenocytes from control mice. Splenocytes from methoxytryptamine - treated mice manifested a lower mitogenic response to CON A (20 %) and a greater mitogenic response to LPS (70 %) than splenocytes from control mice.

Secondly, splenocytes from both melatonin - treated and methoxytryptamine - treated mice produced more γ-interferon and interleukin-2 than splenocytes from control mice. In this regard melatonin had a greater stimulatory effect than methoxytryptamine in the production of γ-interferon and interleukin--2.

Thirdly, when the effects of various crude lymphokines obtained for different groups of mice on a given type of macrophages were investigated, it was found that crude lymphokines from splenocytes of melatonin - treated and methoxytryptamine - treated mice were able to stimulate nitrite production by macrophages (from control mice, melatonin - treated mice, as well as from methoxytryptamine - treated mice) to a greater extent than crude lymphokines from splenocytes of control mice, suggesting that macrophage activating factor was present at higher concentrations in preparations of crude lymphokines from splenocytes of melatonin - treated and methoxytryptamine - treated mice than crude lymphokines from control mice. Crude lymphokines from melatonin-treated mice had a greater stimulatory effect on macrophage nitrite production than those from methoxytryptamine-treated mice, implying that the former contained a higher concentration of macrophage activating factor. It appeared that melatonin exerted a greater stimulatory effect on lymphocytes than methoxytryptamine.

Fourthly, when the effect of a given crude lymphokines was tested on macrophages from different group of mice, it was found that macrophages from melatonin - treated mice produced more nitrite than macrophages from control mice when they were activated by crude lymphokines from melatonin - treated mice or methoxytryptamine - treated mice. Macrophages from methoxytryptamine - treated mice produced the highest nitrite level when compared to macrophages from control mice and melatonin - treated mice, when they were activated by crude lymphokines from control mice, melatonin - treated mice, and methoxytryptamine - treated mice. The data suggest that macrophages from melatonin - treated and methoxytryptamine - treated mice were more sensitive to the action of lymphokines than macrophages from control mice and that methoxytryptamine treatment rendered the macrophages more sensitive to the action of lymphokines than melatonin treatment.

Fifthly, crude lymphokines from melatonin - treated mice but not those from methoxytryptamine - treated mice were able to stimulate greater inhibition of the growth of murine mastocytoma P815 cells by macrophages from control mice (1 - fold), melatonin - treated mice (2.5 - fold) and methoxytryptamine - treated mice (1.3 - fold) when compared to crude lymphokines from control mice, indicating that macrophage activating factor was present at a higher concentration in crude lymphokines from melatonin - treated mice. The same was not true of crude lymphokines from methoxytryptamine - treated mice. This finding again suggests that melatonin exerts a stronger stimulatory effect on lymphocytes than methoxytryptamine.

Sixthly, macrophages from both melatonin - treated and methoxytryptamine - treated mice elicited greater inhibition of the growth of murine mastocytoma P815 cells than macrophages from control mice when they were stimulated by crude lymphokines from melatonin - treated mice, suggesting that macrophages from melatonin - treated and methoxytryptamine - treated mice were more sensitive than macrophages from control mice to crude lymphokines from melatonin - treated mice. Again the higher response observed in macrophage for methoxytryptamine-treated mice suggests that methoxytryptamine exerts a stronger stimulatory effect on macrophages than melatonin. The lack of differential responses among macrophages of the various groups of mice to crude lymphokines from control mice and methoxytryptamine - treated mice may be attributed to the lower activity of macrophage activating factor in these lymphokine preparations.

In summary, treatment with melatonin or methoxytryptamine induced the splenocytes to secrete a higher level of interleukin-2 and γ-interferon which then act on the peritoneal macrophages. The macrophages then respond by secreting nitrite which exerts a killing effect on tumor cells. Melatonin and methoxytryptamine may also act directly on the macrophages to sensitize them to the action of interleukin-2 and γ-interferon.

CON A is known as a T cell mitogen while LPS is known as a B cell mitogen. Splenocytes from melatonin - treated mice showed a greater mitogenic response in both T cell and B cell populations when compared to splenocytes from control mice. Splenocytes from methoxytryptamine - treated mice showed a lower but not

significant different mitogenic response in T cell population and a higher mitogenic response in B cell populations when compared to splenocytes from control mice. It was important to the animals since the spleen is an important lymphoid organ. Lymphocytes with a greater response to stimulants are expected to acquire higher cell - mediated and humoral - immune response. It was further proved by the fact that splenocytes from melatonin - treated mice produced more γ-interferon and interleukin - 2 than splenocytes from control mice. Splenocytes from methoxytryptamine - treated mice exerted a smaller enhancing effect in the same parameters when compared to splenocytes from melatonin - treated mice. However, the lack of a stimulatory effect of methoxytryptamine on the mitogenic response to CON A is enigmatic.

Interferons are proteins (Friedman 1981) that induce an antiviral state in animal cells and also other biological activities. γ - interferon is secreted mainly by T lymphocytes. It stimulates macrophages and natural killer cells. It first inhibits and later enhances antibody formation by B lymphocytes. Mixed lymphocytes culture is a measure of function T lymphocytes. γ - interferon is known to decrease the mixed lymphocytes reaction, which may be due to the negative feedback mechanism of the cytokine. The augmented generation of γ-interferon following melatonin and methoxytryptamine treatment points to an immunostimulatory action of the pineal indoles.

Interleukin - 2 is also known as a T cell growth factor, which is widely used to maintain the growth of the murine tumor - specific cytotoxic T cell lines (Gillis

1978). Cytotoxic T cells are important effector cells other than macrophages which maintain the cell - mediated immune response of the immune system. Besides, pre-culture with interleukin - 2 enhanced the cytotoxic activity of peripheral blood or spleen cell populations. The activated cells are termed lymphokine activated killer cells. Their precursor cells are indistinguishable from natural killer cells so they may not represent separate cell lineage. Interleukin - 2 has also been reported (Strieter 1989) to induce tumor necrosis factor - α gene expression in human alveolar macrophages and blood monocytes. Lewinski (1989) reported that melatonin suppressed natural killer cells in vitro. Results of the present study are at variance with his finding because melatonin and methoxytryptamine treatments resulted in elevated production of interleukin-2 by splenocytes.

However, it was surprising that macrophages from methoxytryptamine - treated mice exhibited a greater response to stimulation than macrophages from melatonin - treated mice. Actually, one of the macrophage activating factors is γ-interferon. Macrophage activation is known to require the action of γ-interferon. Taramelli (1981) found that LPS was absolutely required for macrophage activation in the murine mastocytoma P815 microcytotoxicity test. Ding (1988) tested the ability of twelve different cytokines including α - interferon, β- interferon, γ - interferon, TNF - α, TNF - β, CSF - GM, CSF - M, interleukin - 1β, interleukin - 2, interleukin - 3, interleukin - 4, and TGF - β to induce nitrite and hydrogen peroxide production by macrophages. He found that γ - interferon was the only cytokine capable of inducing both nitrite and hydrogen peroxide release. He also found that the arginine analog, N^G-monomethylarginine, inhibited nitrite release but not hydrogen peroxide

production. Nitrite is important for macrophages to carry out its cytotoxic functions. L - arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells (Hibbs 1987). These metabolic changes include inhibition of mitochondrial respiration such as activities of NADH : ubiquinone oxidoreductase and succinate : ubiquinone oxidoreductase, inhibition of aconitase in citric acid cycle, and inhibition of incorporation of ^{3}H-thymidine into the DNA. Nitrate and nitrite, also known as reactive nitrogen intermediates, are the metabolites of the L - arginine (Hibbs 1987). Inhibition of the growth of murine mastocytoma P815 cells was the result of an action of the reactive nitrogen intermediates produced by the peritoneal macrophages after stimulation by macrophage activating factor (e.g. γ-interferon) secreted by the splenocytes. The present study revealed a disparity in the immunoregulatory actions of melatonin and methoxytryptamine although similarities do exist. Differences were observed in the mitogenic response to CON A and in the activity of macrophage activating factor secreted by the splenocytes. The similarities in action point to an immunostimulatory role of the two pineal indoles but it appears that melatonin exerts a stronger stimulatory effect on lymphocytes while methoxytryptamine exerts a stronger stimulatory effect on macrophages. It is noteworthy that differences between the actions of melatonin and methoxytryptamine have already been detected in chapter 3.

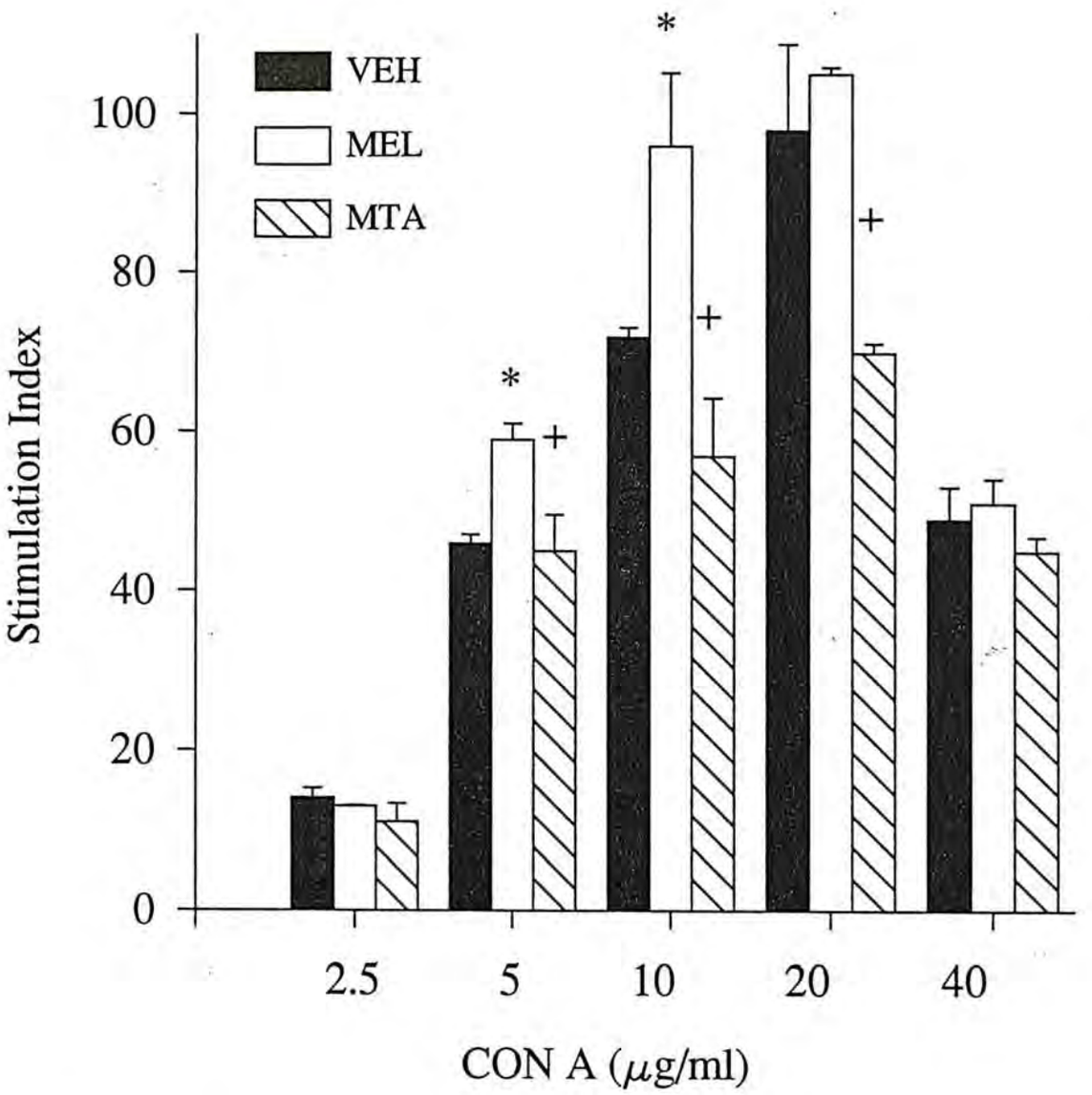

Figure 4.1 Mitogenic response of mouse splenocytes to CON A. The mice (N = 20/group) had been treated with melatonin and methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks. Results are expressed as means ± standard deviation (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from corresponding melatonin group. VEH = control, MEL = melatonin, MTA = methoxytryptamine, CON A = concanavalin A.

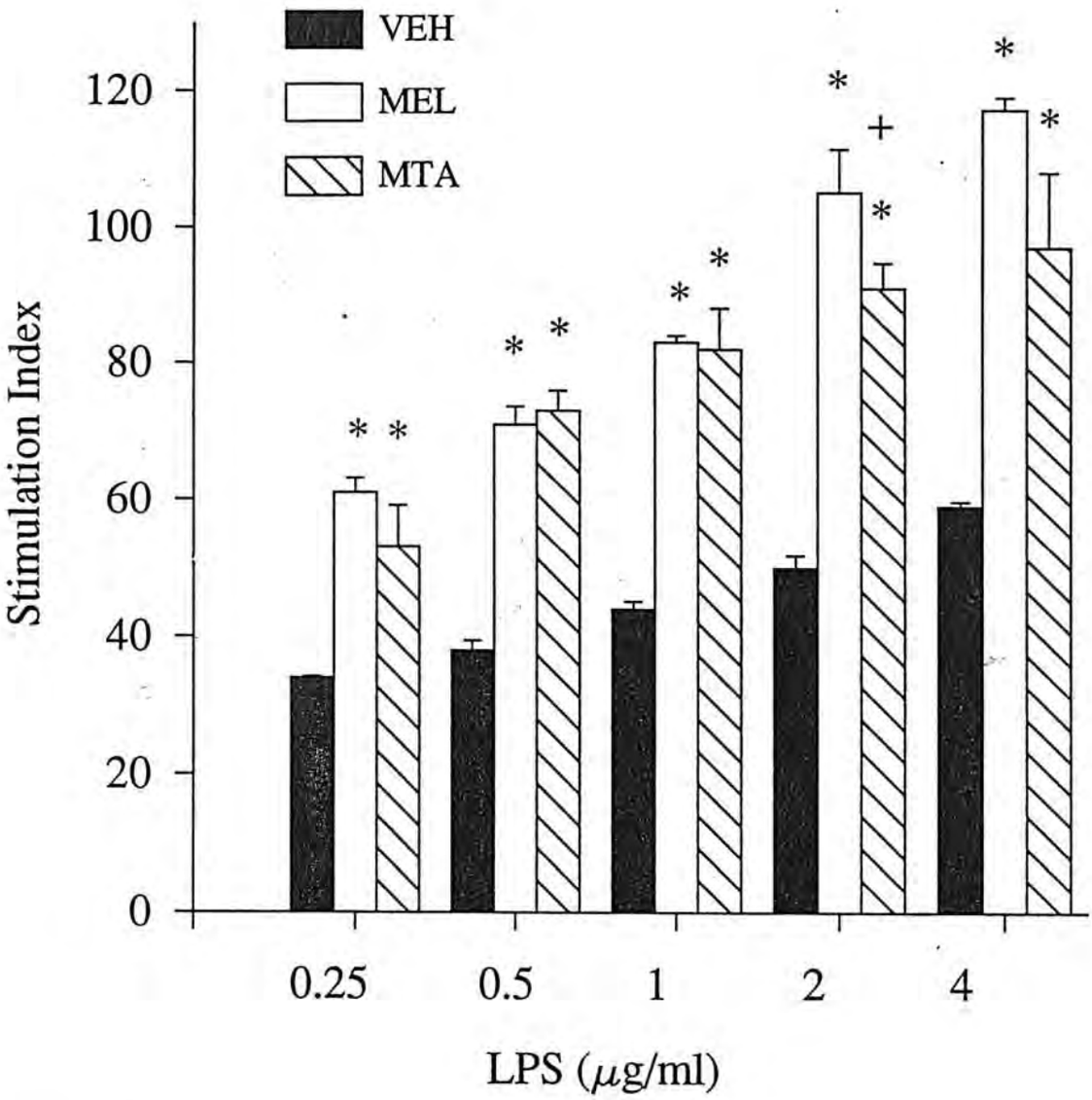

Figure 4.2 Mitogenic response of mouse splenocytes to LPS. The mice (N = 20/group) had been treated with melatonin and methoxytryptamine in drinking water (100 μg/ml) for 2 weeks. Results are expressed as means ± standard deviation (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from corresponding melatonin group. VEH = control, MEL = melatonin, MTA = methoxytryptamine, LPS = lipopolysaccharide.

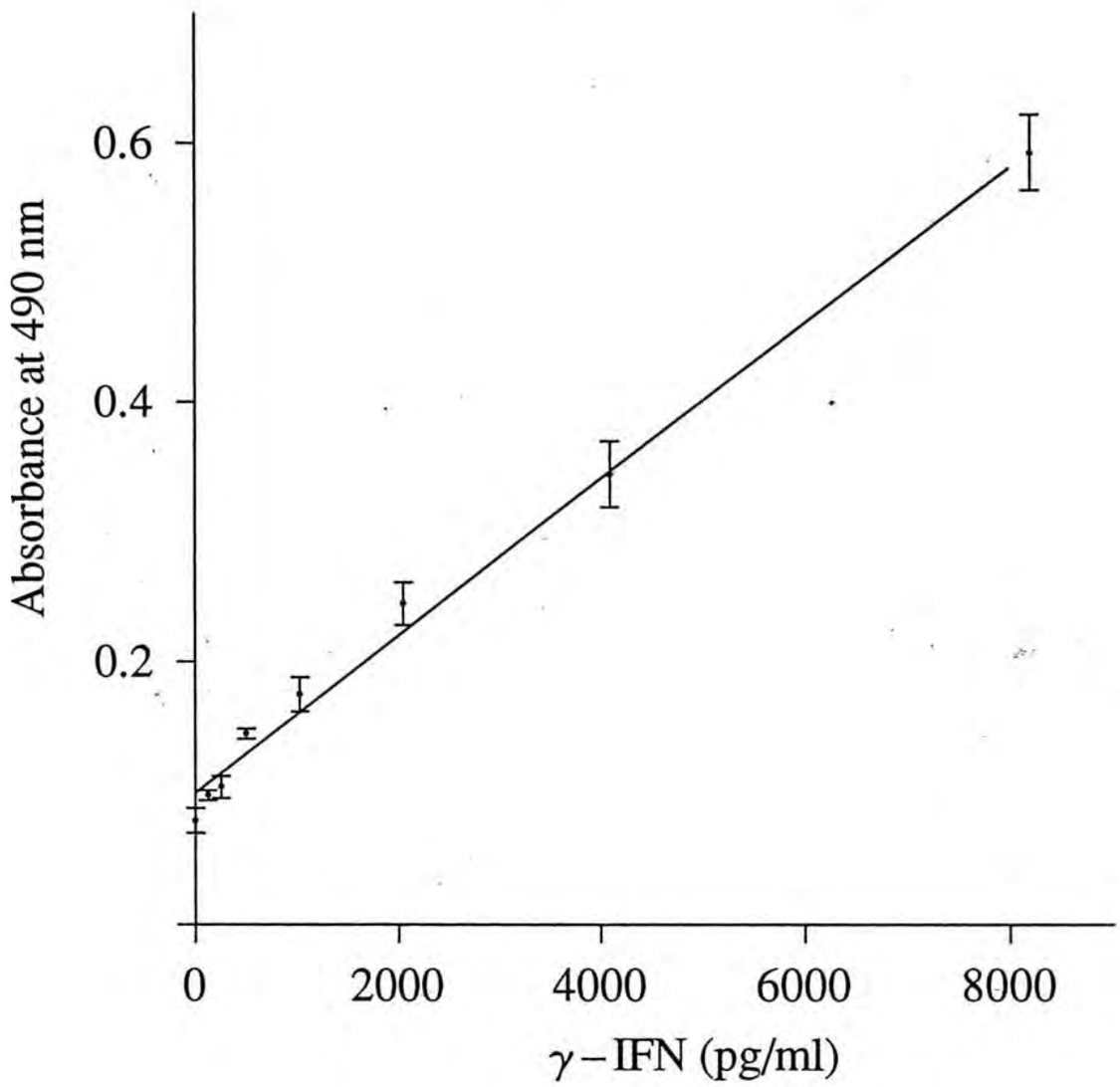

Figure 4.3 Standard curve for γ-interferon assay

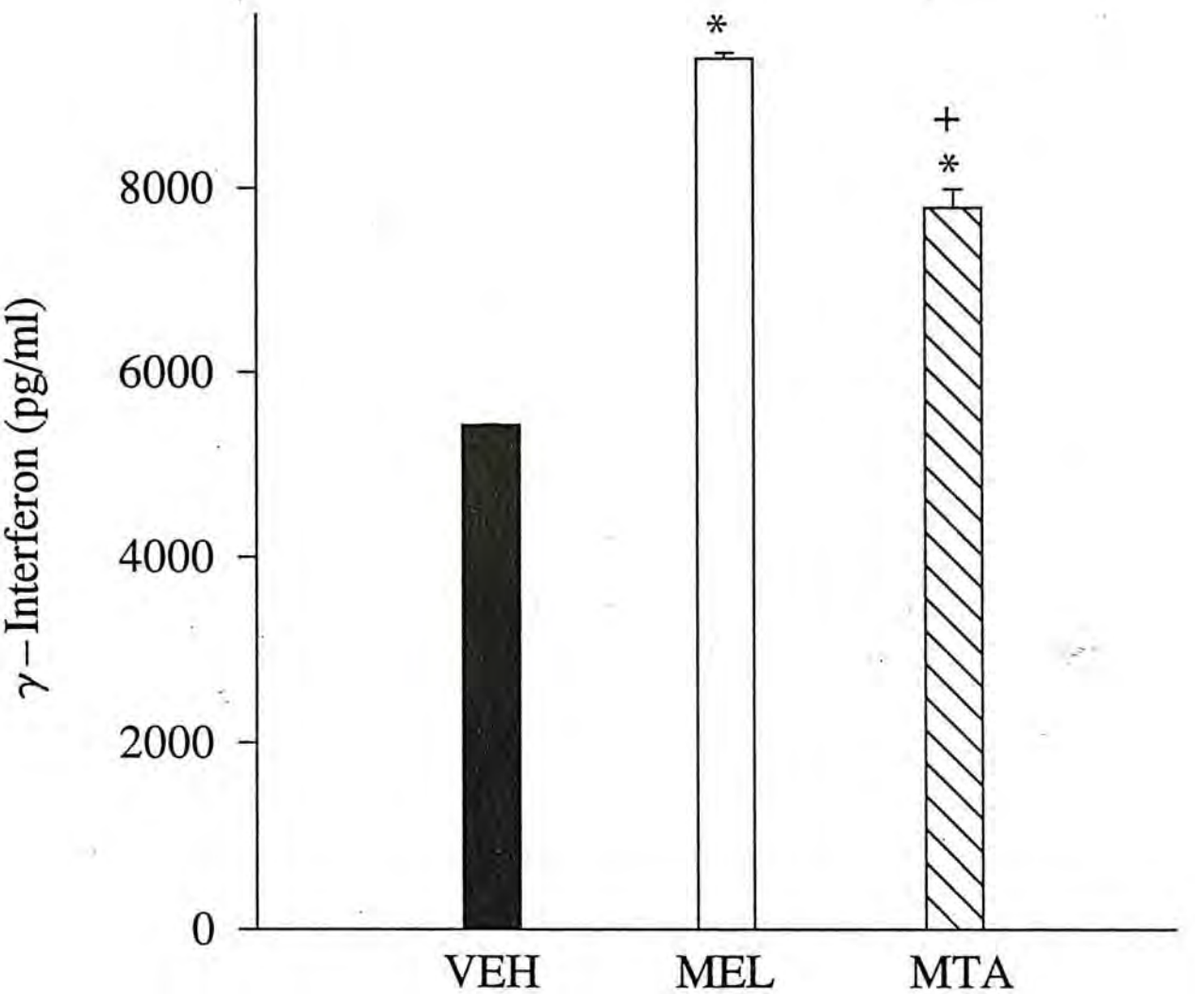

Figure 4.4 In vitro production of γ-interferon by mouse splenocytes from mice (N = 20/group) which had been treated with melatonin and methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks. Results are expressed as means ± standard deviation (n = 2). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from melatonin group. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

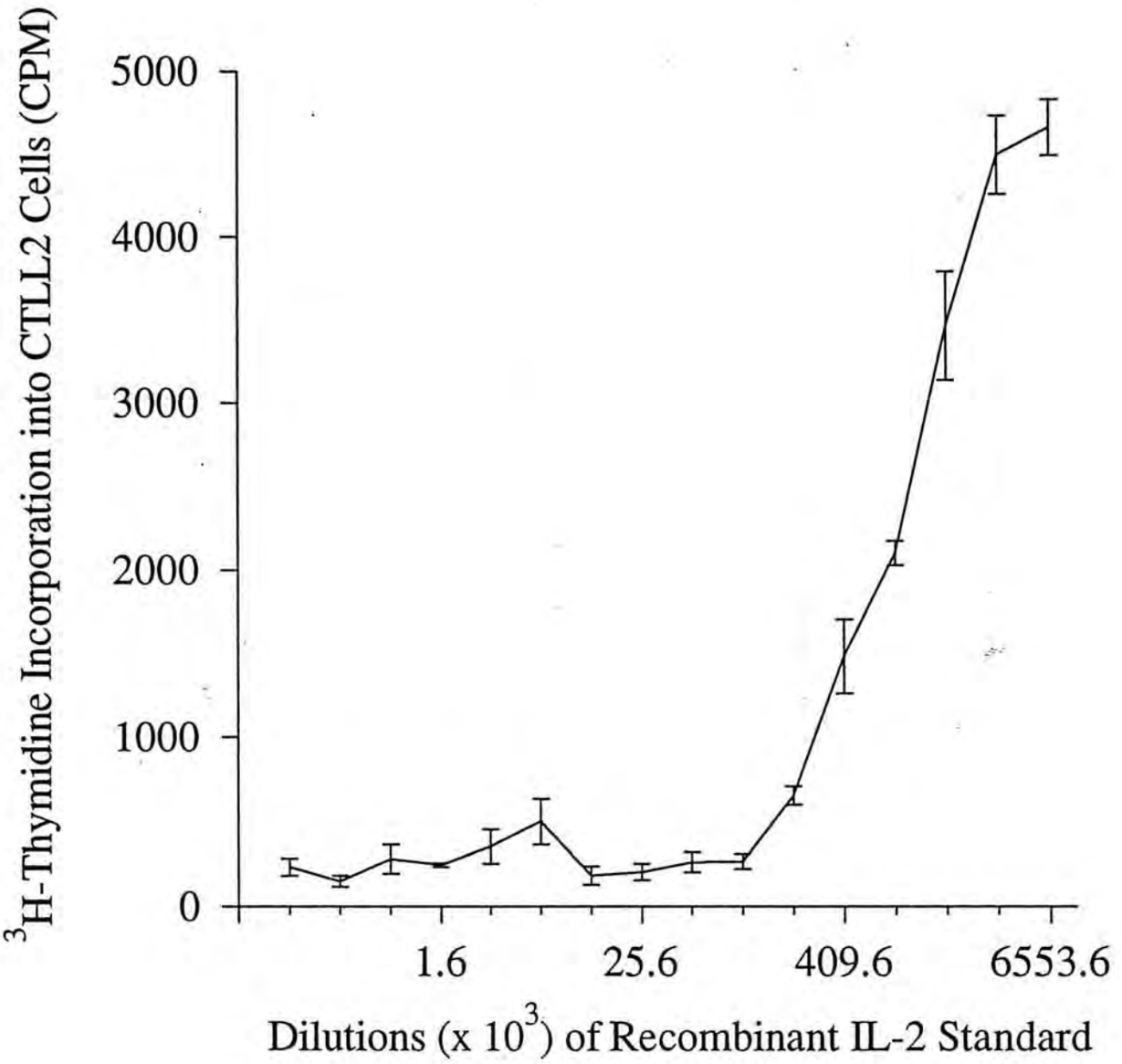

Figure 4.5 Standard curve for recombinant interleukin-2 (IL-2) assay using CTLL 2 cells.

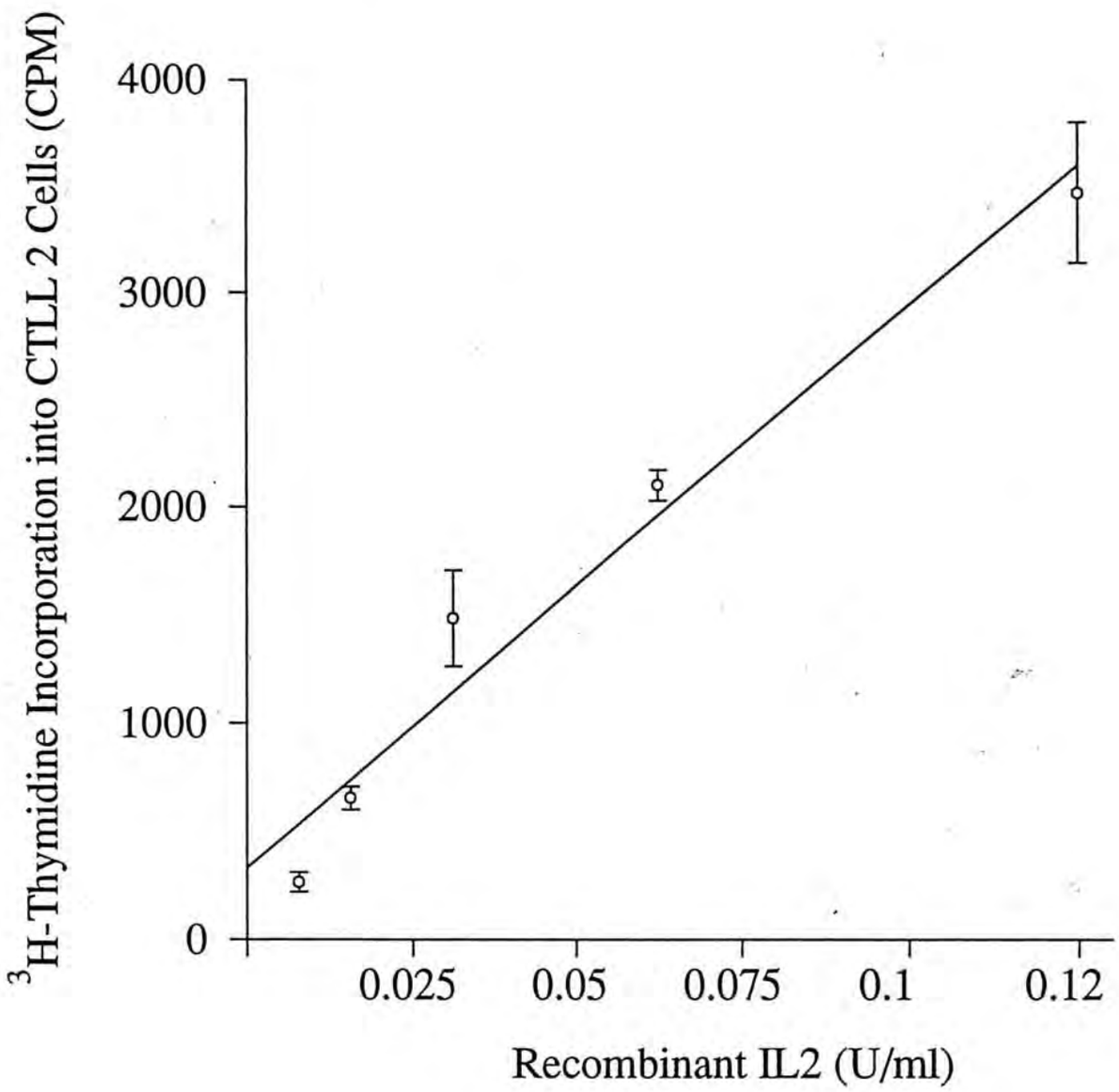

Figure 4.6 Linear portion of standard curve for recombinant interleukin-2 (IL-2) assay using CTLL 2 cells.

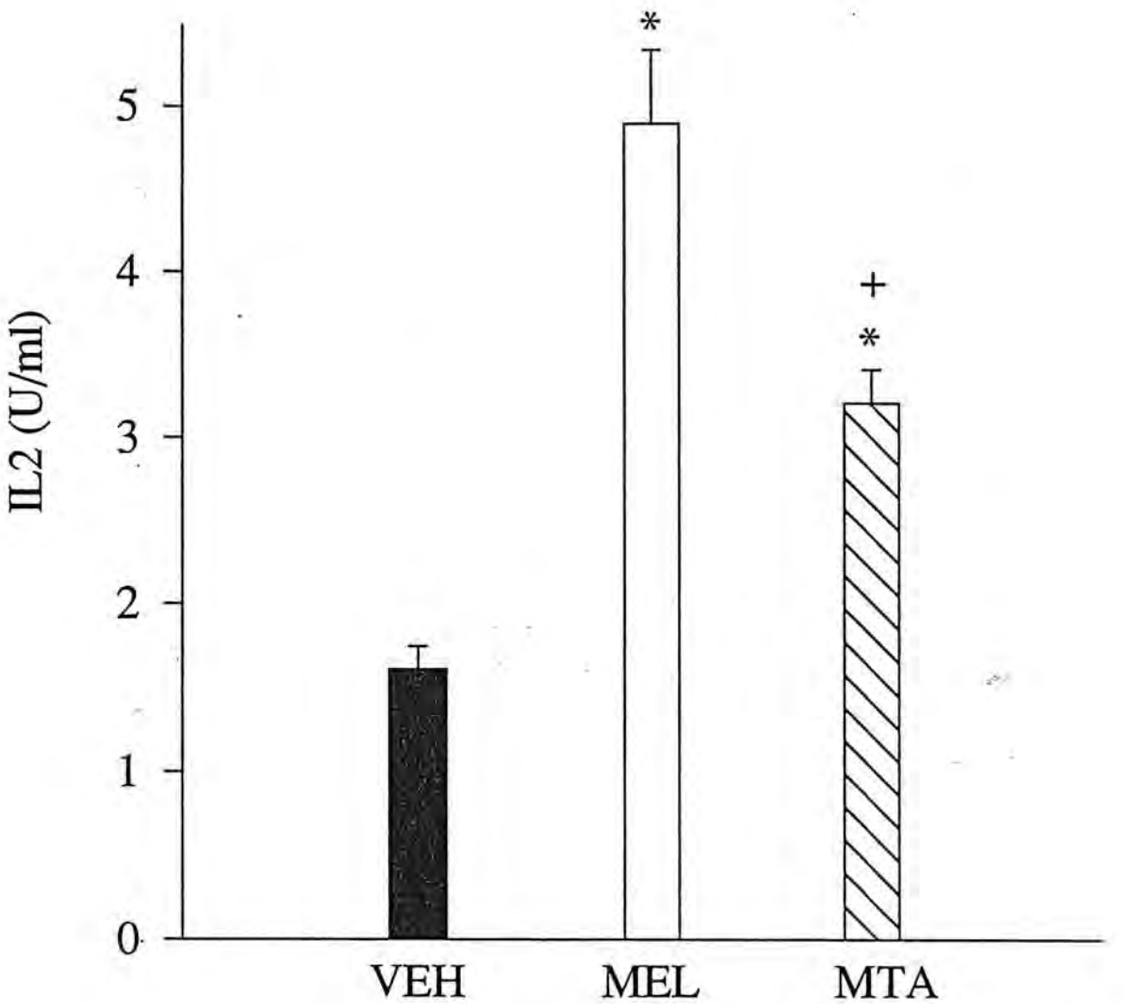

Figure 4.7 In vitro production of interleukin-2 by splenocytes from mice (N = 20/group) which had been treated with melatonin and methoxytryptamine in drinking water (100 μg/ml) for 2 weeks. Results are expressed as means ± standard deviation (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from melatonin group. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

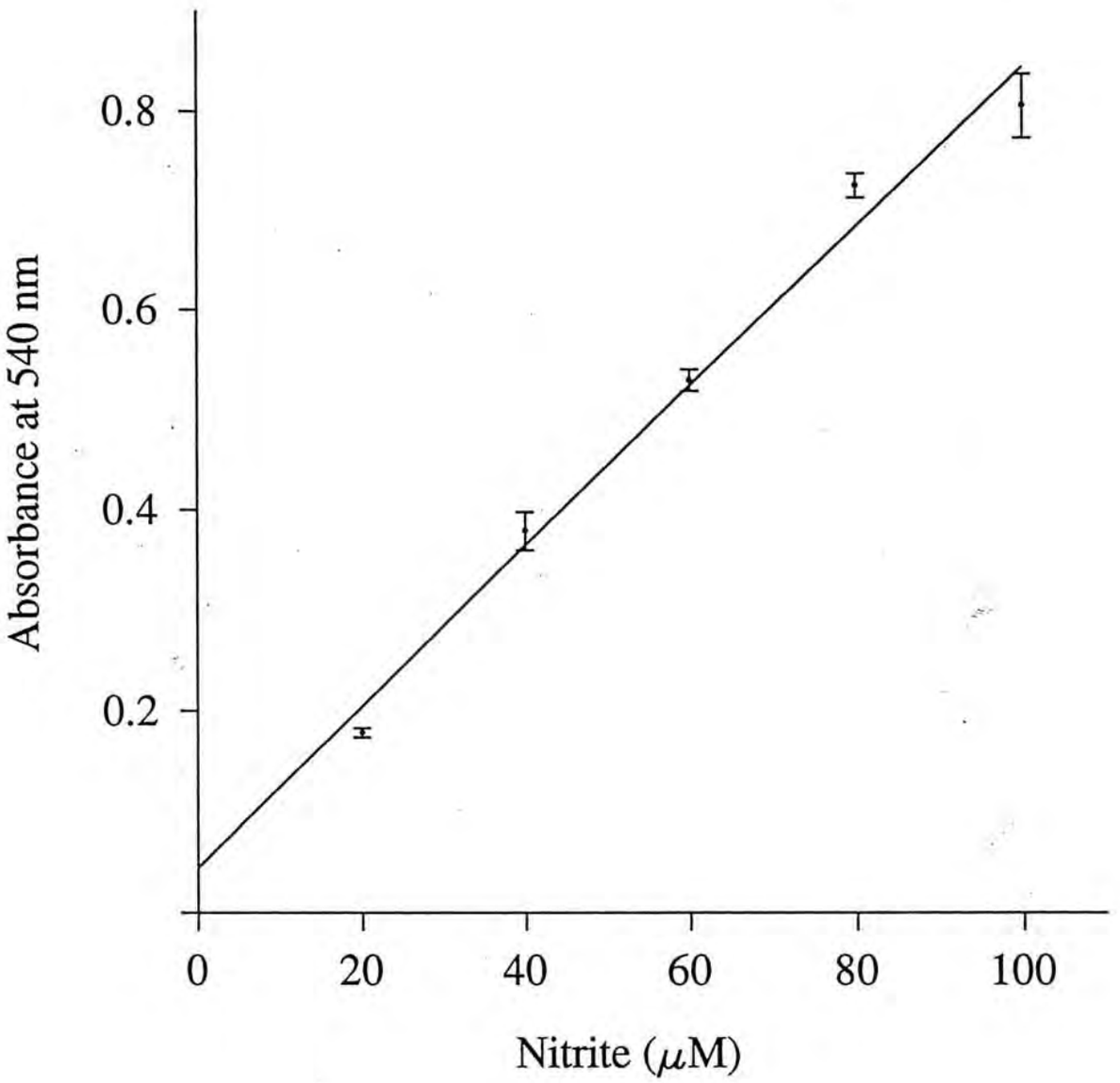

Figure 4.8 Standard curve for nitrite assay using sodium nitrite as standard.

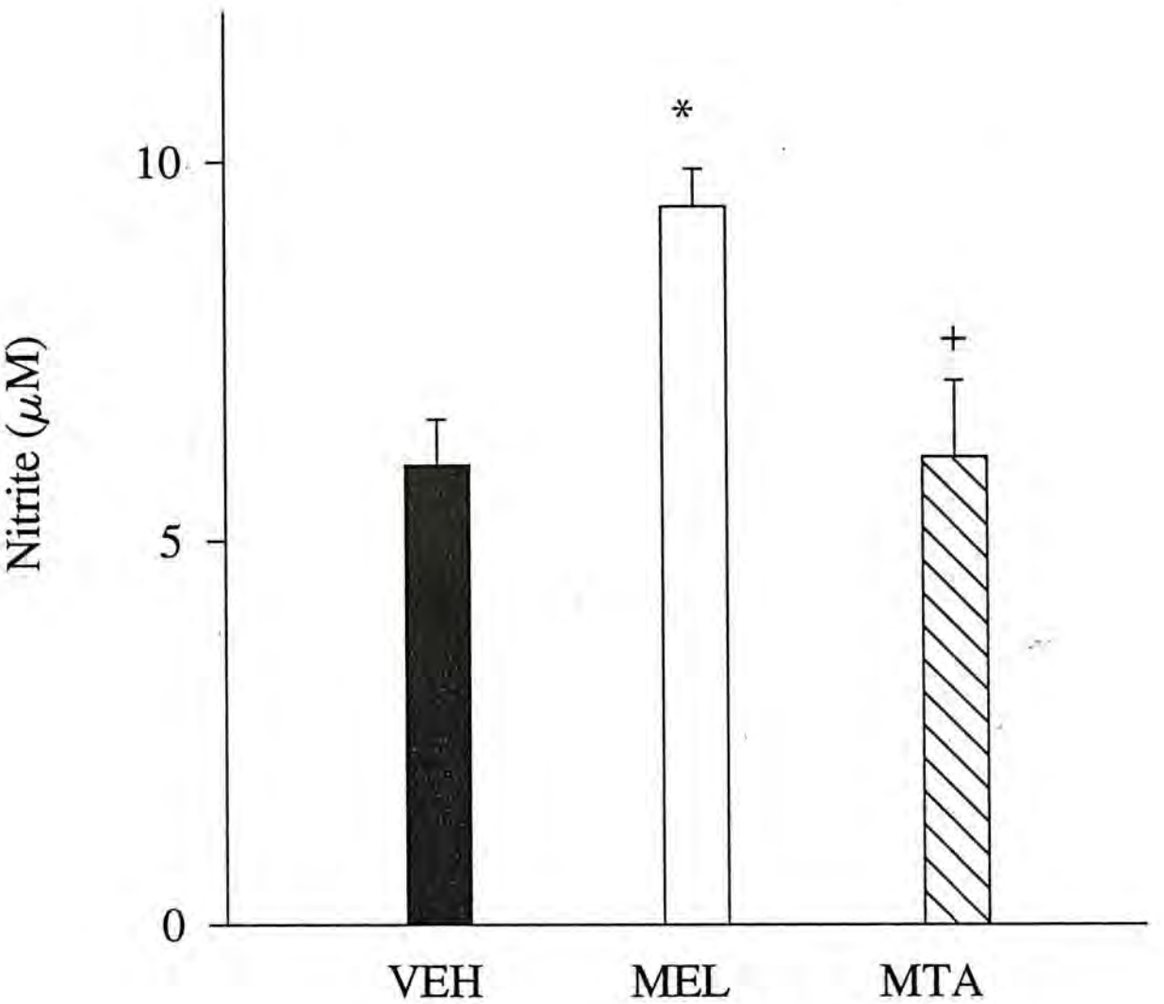

Figure 4.9 In vitro nitrite production by peritoneal macrophages of control mice (N = 20/group) after stimulation by crude lymphokines from mice (N = 20/group) which had been treated with melatonin and methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks. Results are expressed as means ± standard deviation (n = 4). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from melatonin group. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

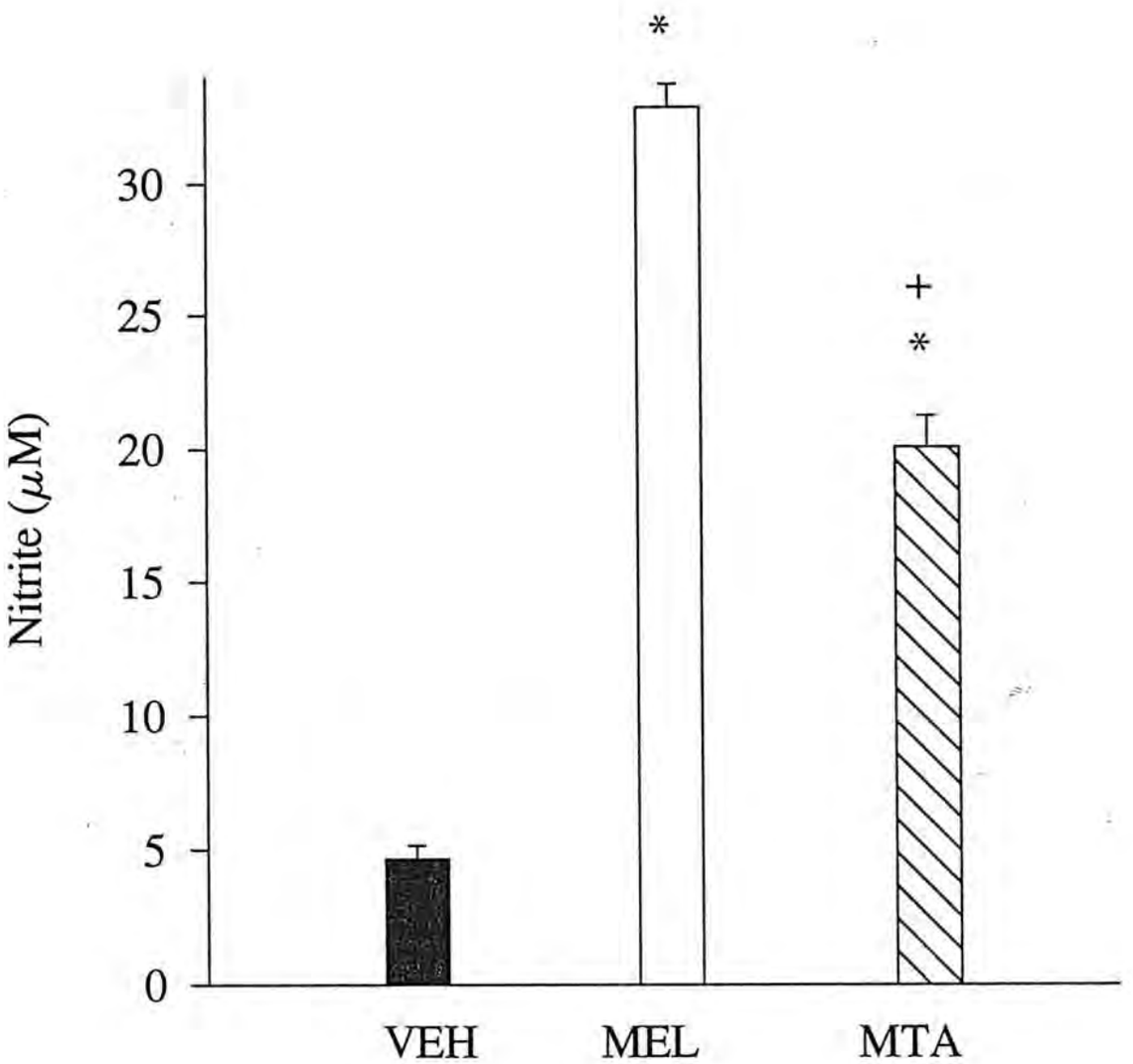

Figure 4.10 In vitro nitrite production by peritoneal macrophages of melatonin - treated mice (N = 20/group) after stimulation by crude lymphokines from mice (N = 20/group) which had been treated with melatonin and methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks. Results are expressed as means ± standard deviation (n = 4). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from melatonin group. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

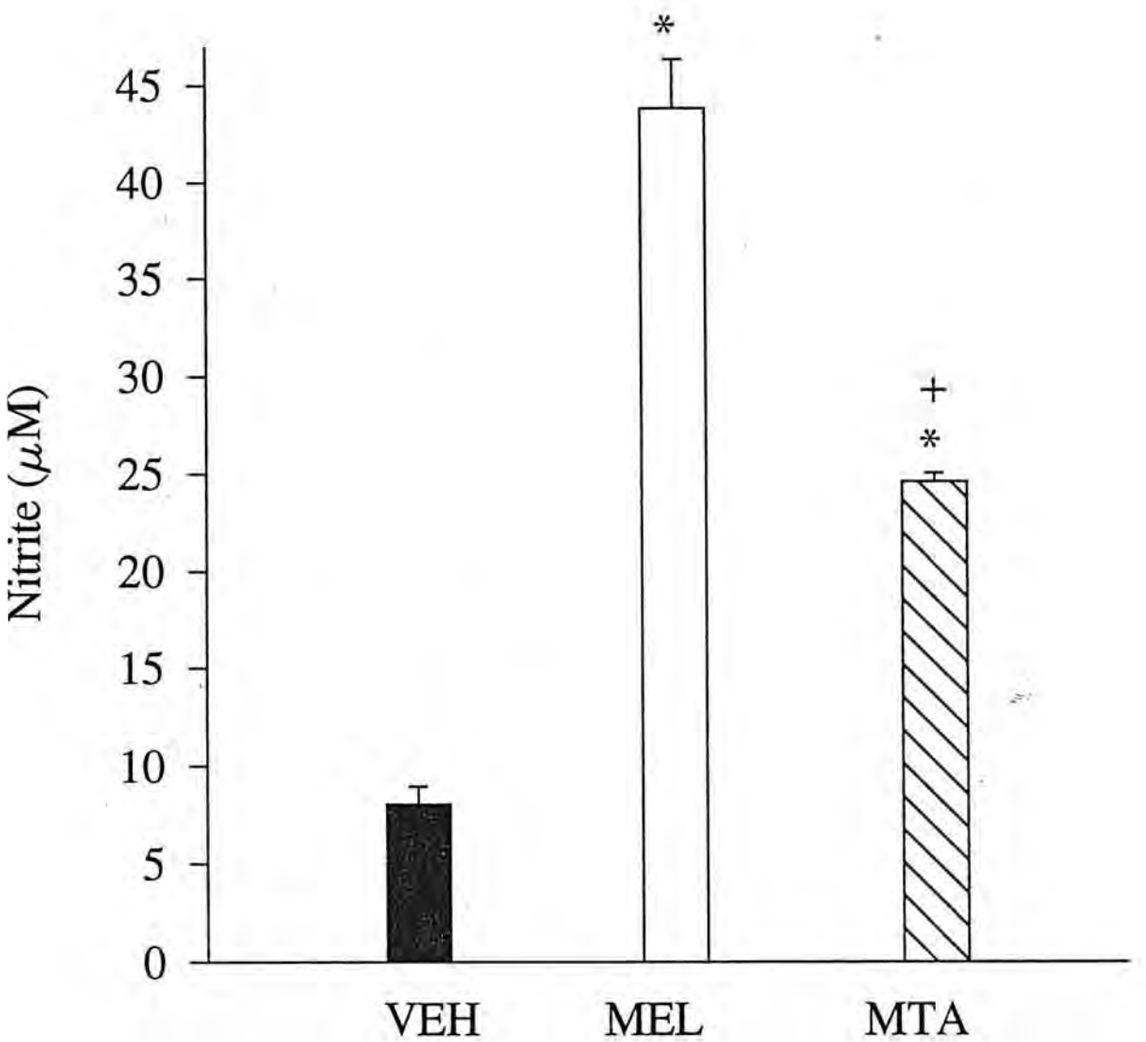

Figure 4.11 In vitro nitrite production by peritoneal macrophages of methoxytryptamine - treated mice (N = 20/group) after stimulation by crude lymphokines from mice (N = 20/group) which had been treated with melatonin and methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks. Results are expressed as means ± standard deviation (n = 4). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from melatonin group. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

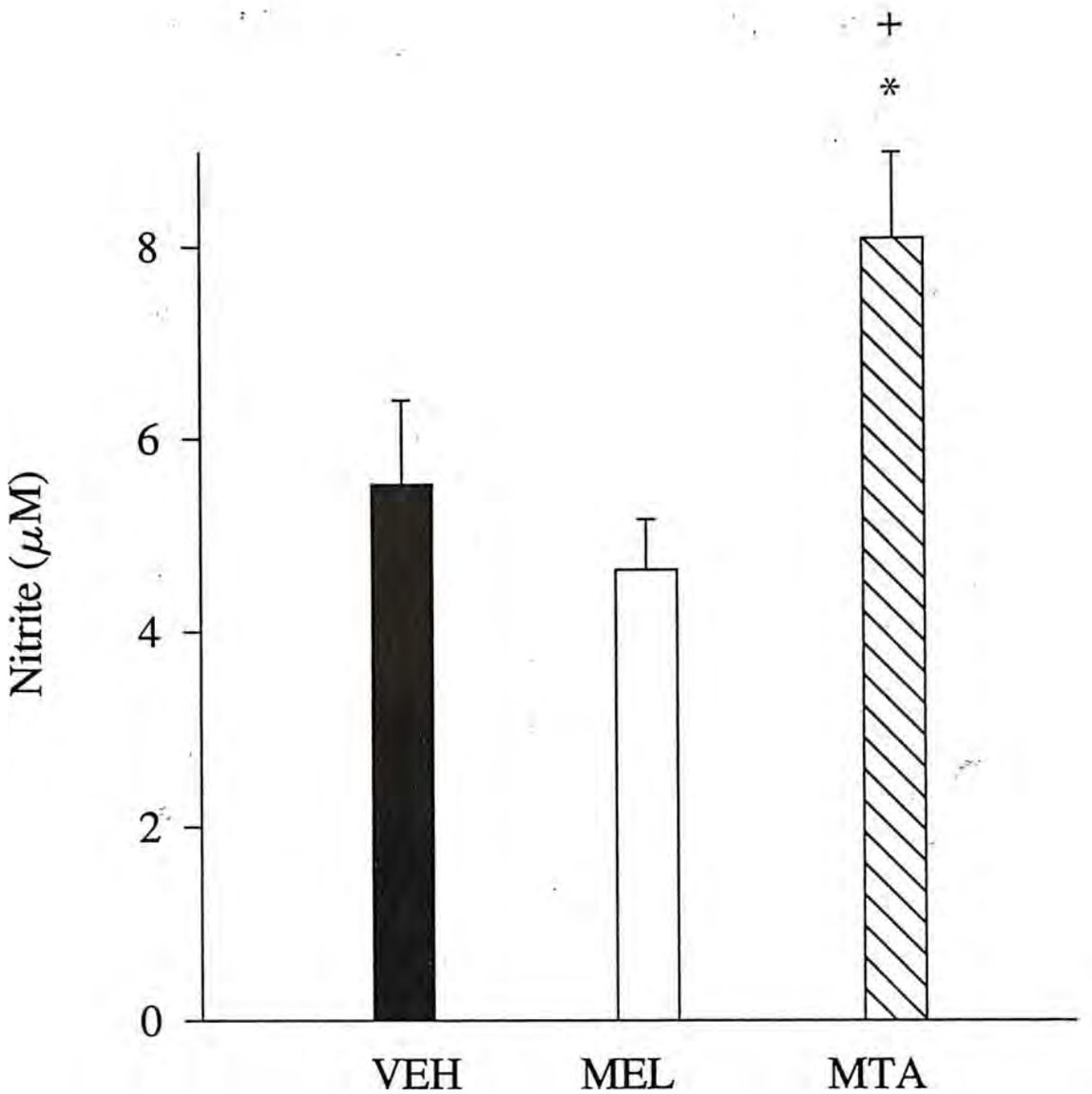

Figure 4.12 In vitro nitrite production by peritoneal macrophages from mice (N = 20/group) which had been treated with melatonin and methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks. The macrophages had been subjected to in vitro stimulation by crude lymphokines from control mice (N = 20/group). Results are expressed as means ± standard deviation (n = 4). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from melatonin group. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

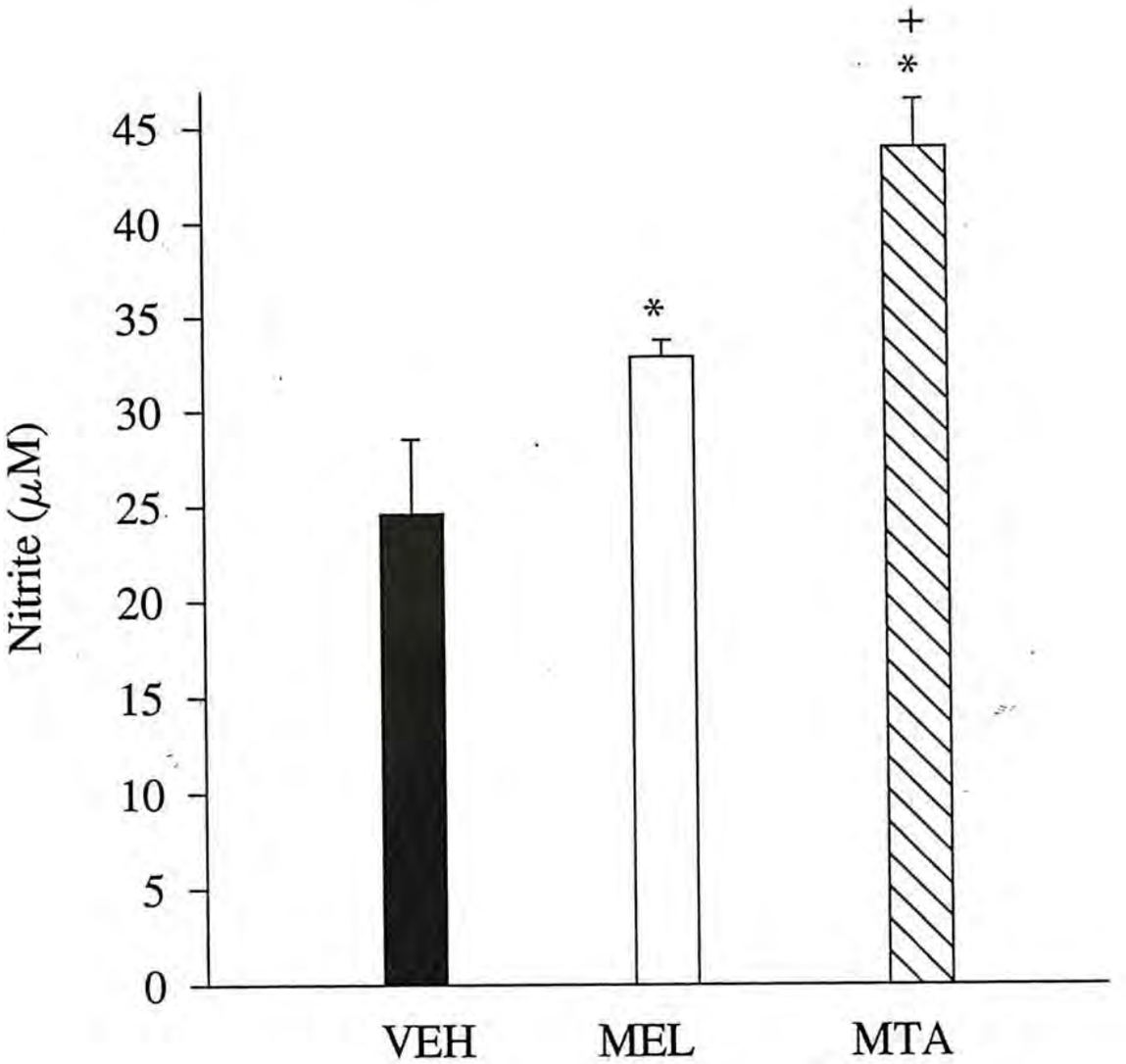

Figure 4.13 In vitro nitrite production by peritoneal macrophages from mice (N = 20/group) which had been treated with melatonin and methoxytryptamine in the drinking water (100 μg/ml) for 2 weeks. The macrophages had been subjected to in vitro stimulation by crude lymphokines from melatonin - treated mice (N = 20/group). Results are expressed as means ± standard deviation (n = 4). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from melatonin group. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

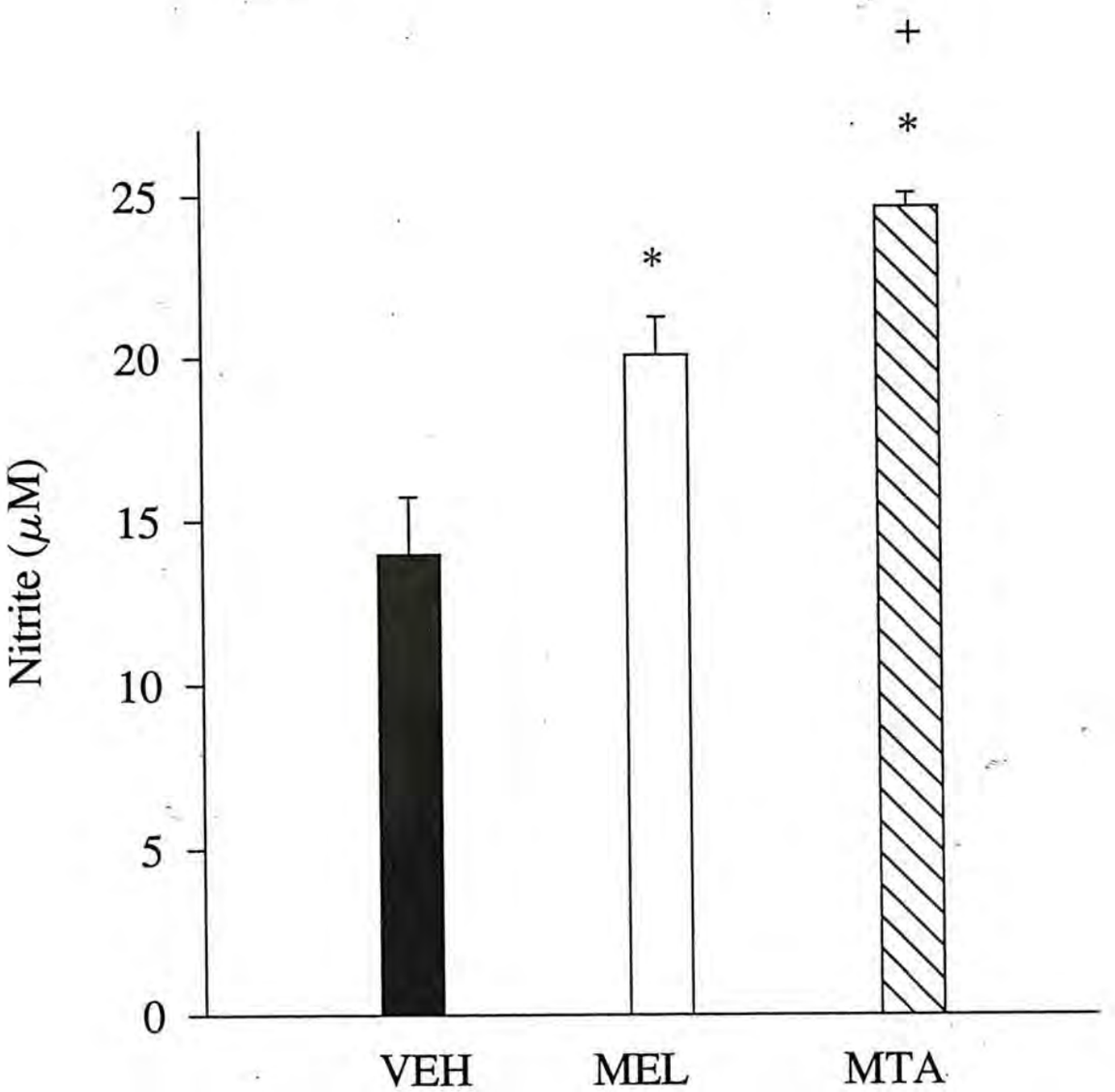

Figure 4.14 In vitro nitrite production by peritoneal macrophages from mice (N = 20/group) treated with melatonin and methoxy - tryptamine in the drinking water (100 μg/ml) for 2 weeks. The macrophages had been subjected to in vitro stimulation by crude lymphokines from methoxy - tryptamine - treated mice (N = 20/group). Results are expressed as means ± standard deviation (n = 4). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from melatonin group. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

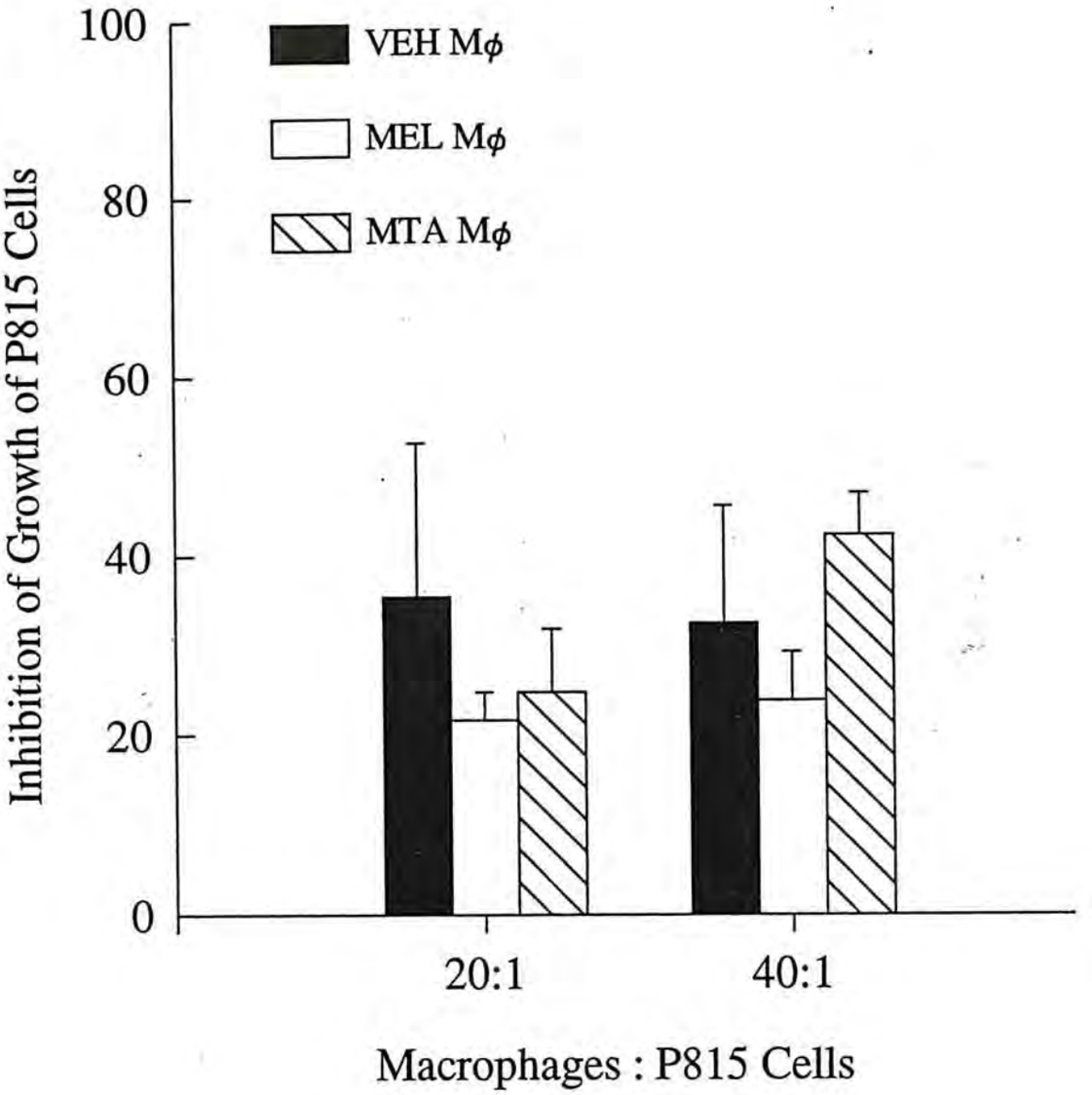

Figure 4.15 In vitro killing of murine mastocytoma P815 cells by peritoneal macrophages of mice which have been treated with melatonin or methoxytryptamine in the drinking water for 2 weeks. The macrophages have been subjected to in vitro stimulation by crude lymphokines from control mice. Results are expressed as means ± standard deviation (n = 3). VEH = control, MEL = melatonin, MTA = methoxytryptamine.

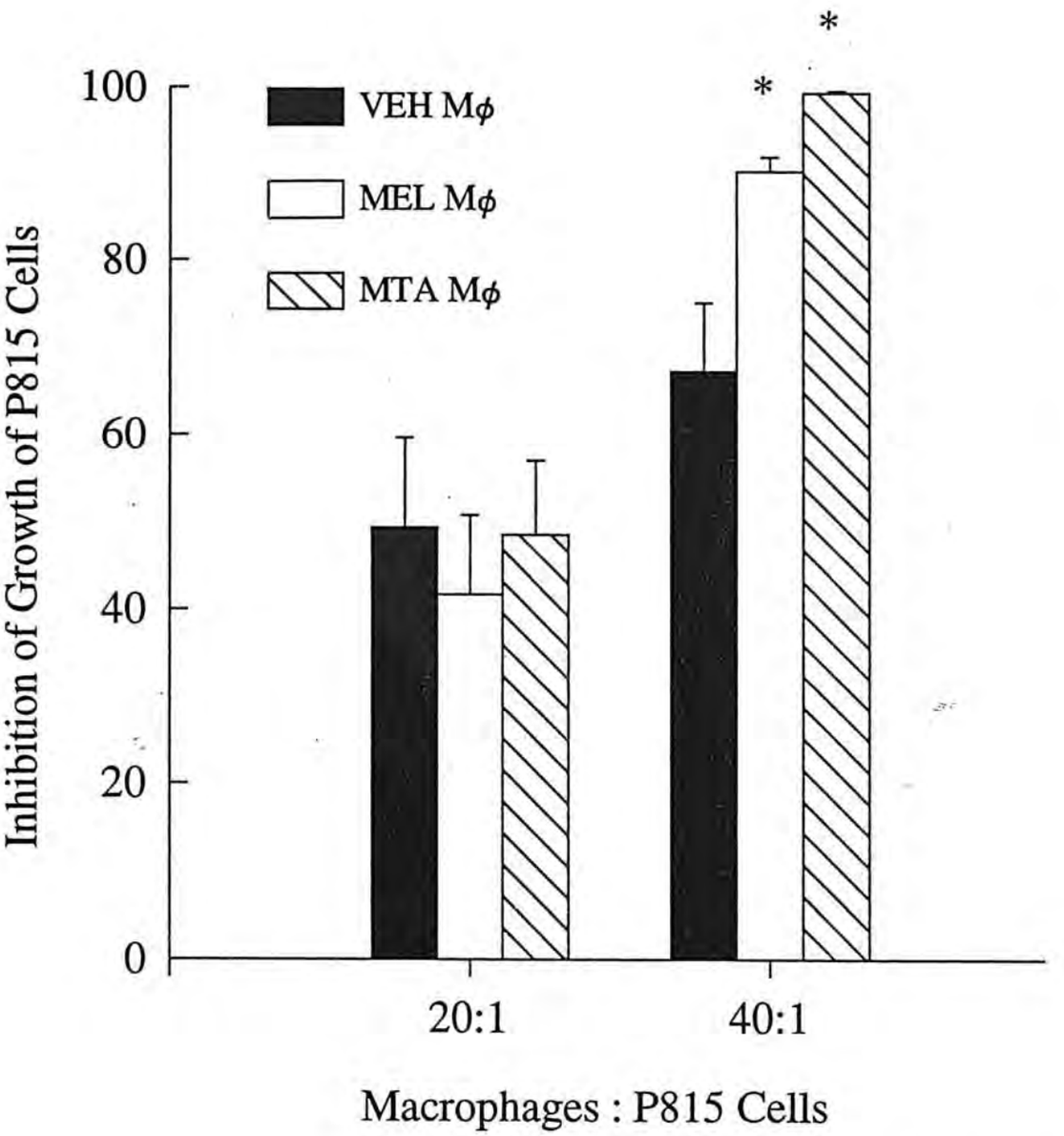

Figure 4.16 In vitro killing of murine mastocytoma P815 cells by peritoneal macrophages of mice (N = 20/group) treated with melatonin or mehtoxytryptamine in the drinking water (100 µg/ml) for 2 weeks. The macrophages had been subjected to in vitro stimulation by crude lymphokines from melatonin - treated mice (N = 20/group). Results are expressed as means ± standard deviation (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

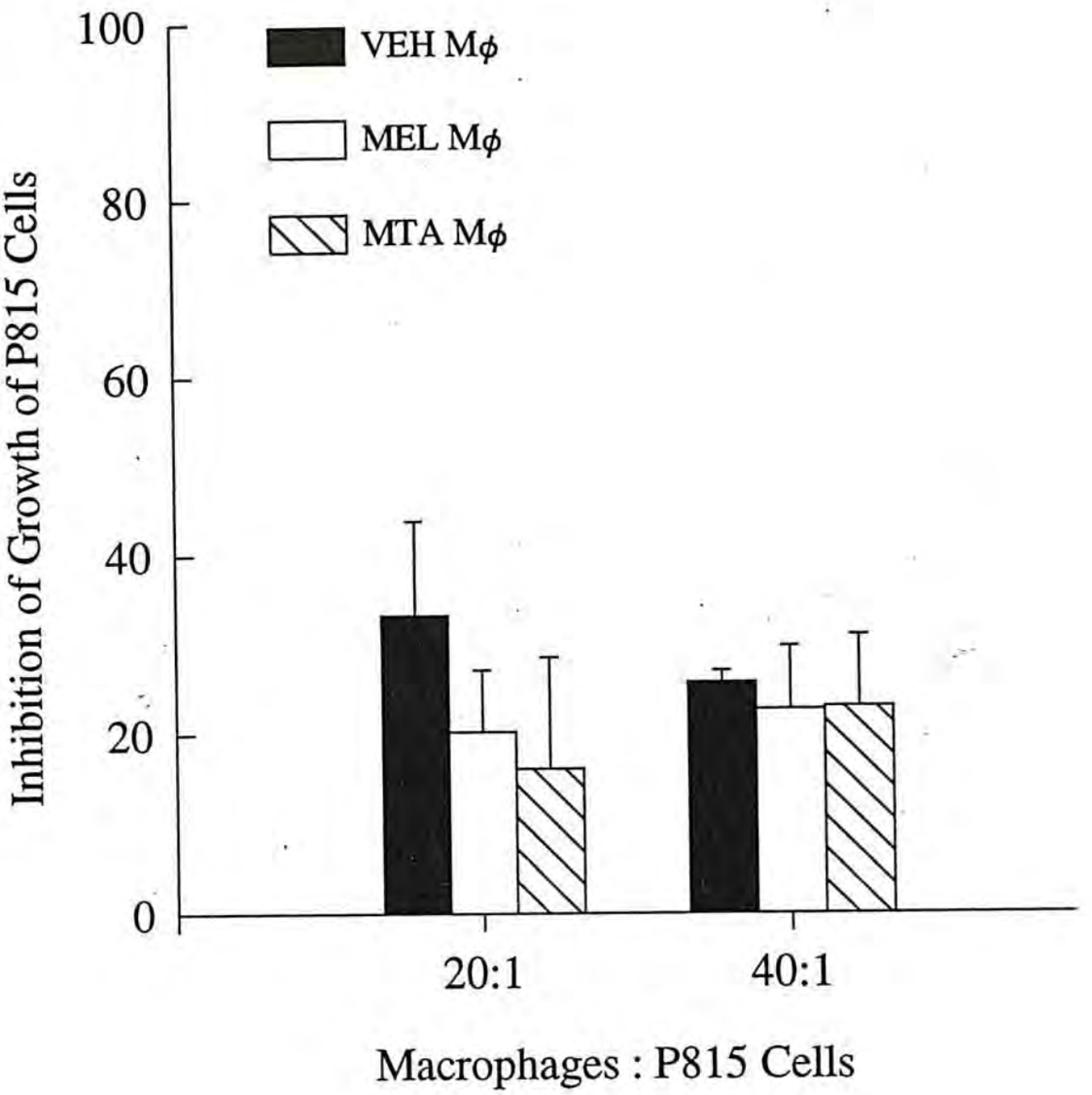

Figure 4.17 In vitro killing of murine mastocytoma P815 cells by peritoneal macrophages of mice (N = 20/group) treated with melatonin or methoxytryptamine in the drinking water (100 µg/ml) for 2 weeks. The macrophages had been subjected to in vitro stimulation by crude lymphokines from methoxytryptamine - treated mice (N = 20/group). Results are expressed as means ± standard deviation (n = 3). VEH = control, MEL = melatonin, MTA = methoxy - tryptamine.

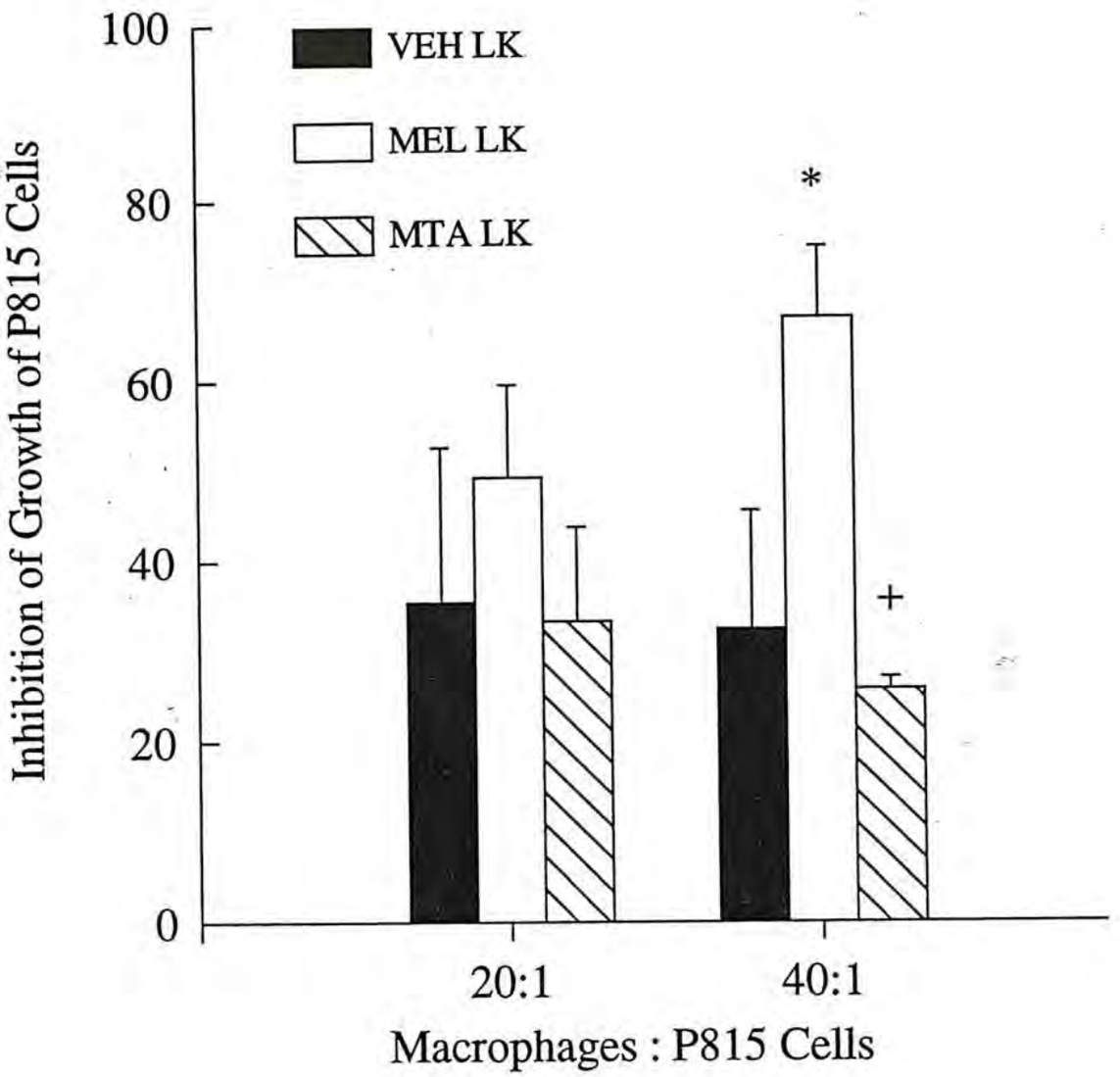

Figure 4.18 In vitro killing of murine mastocytoma P815 cells by peritoneal macrophages of control mice (N = 20/group) after in vitro stimulation by crude lymphokines from melatonin - treated or methoxytryptamine - treated mice (N = 20/group). Results are expressed as means ± standard deviation (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from melatonin group. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

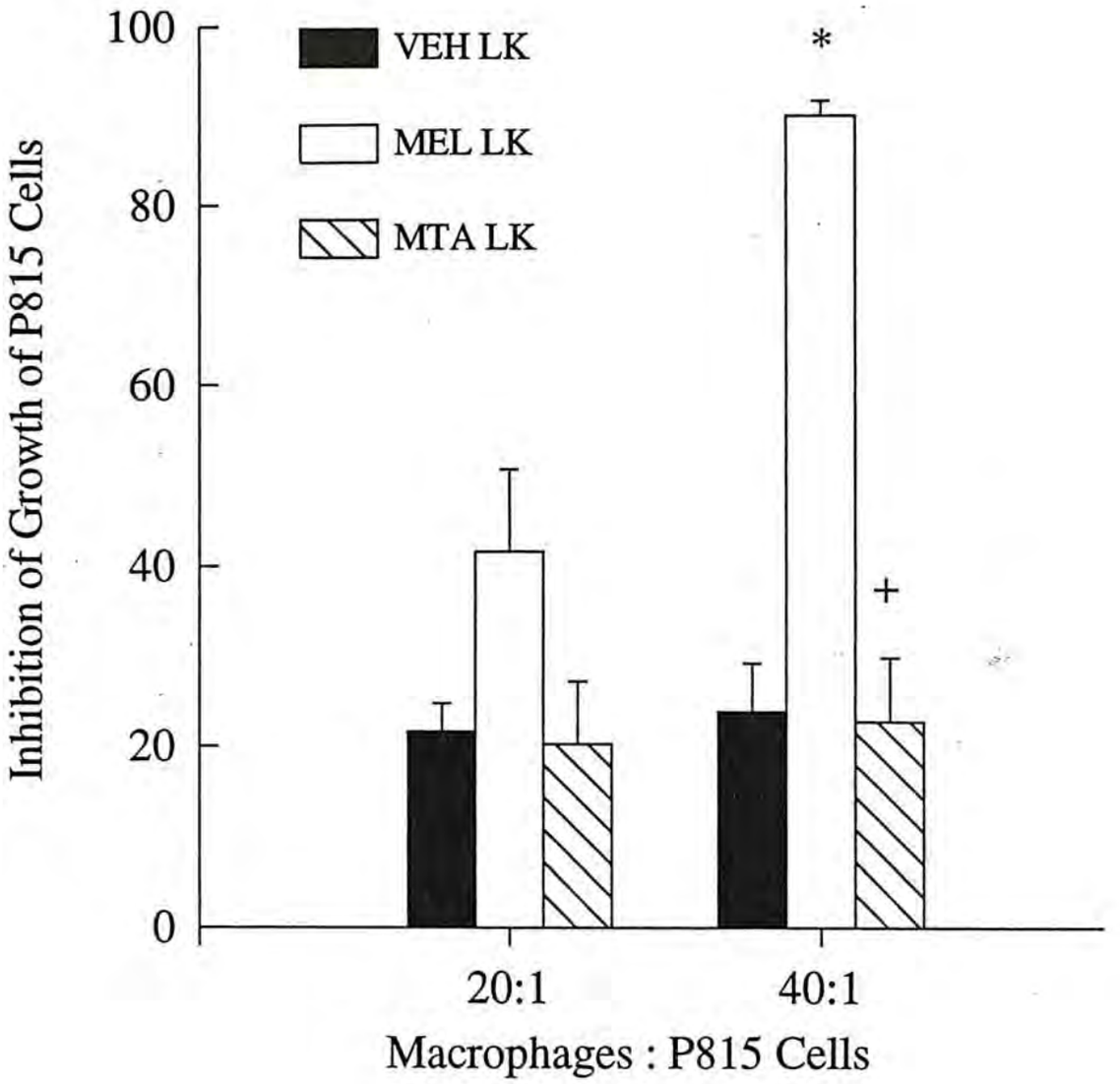

Figure 4.19 In vitro killing of murine mastocytoma P815 cells by peritoneal macrophages from melatonin - treated mice (N = 20/group) after in vitro by crude lymphokines from melatonin - treated or methoxytryptamine - treated mice (N = 20/group). Results are expressed as means ± standard deviation (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from melatonin group. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

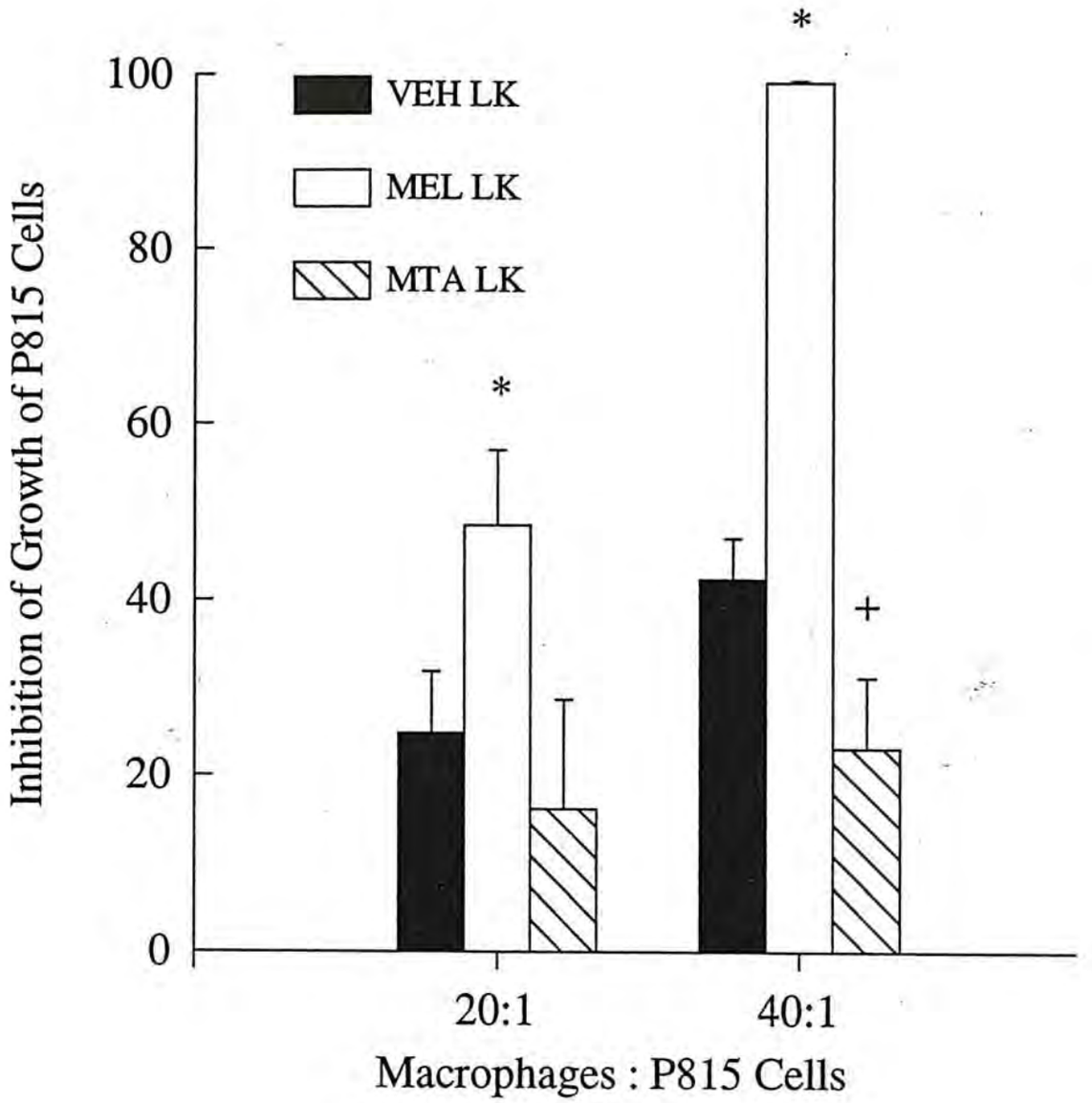

Figure 4.20 In vitro killing of murine mastocytoma P815 cells by peritoneal macrophages from methoxytryptamine - treated mice (N = 20/group) after in vitro stimulation by crude lymphokines from melatonin - treated or methoxytryptamine - treated mice (N = 20/group). Results are expressed as means ± standard deviation (n = 3). An asterisk (*) denotes a statistically significant difference ($p < 0.05$) from control. A cross (+) denotes a statistically significant difference ($p < 0.05$) from melatonin group. VEH = control, MEL = melatonin, MTA = methoxytryptamine.

Chapter 5 General Discussion

Clinical trials of melatonin therapy in metastatic patients have been done recently in Italy. Lissoni (1989) treated metastatic cancer patients who did not respond to standard therapies, with intramuscular injections of melatonin (20 mg daily) followed by oral administration of melatonin (10 mg daily) in a maintenance period after remission. Eight of the fourteen patients showed improvement in their performance status. The T4/T8 ratio of in patients who did not show progress was significantly higher after therapy than before therapy but was still lower than the ratio in patients who showed progress. The serum levels of growth hormone, somatomedin-C, and ß-endorphin were not affected by melatonin administration. Based upon his finding and the fact that the biochemical basis of melatonin therapy has not been documented, he suggested that melatonin therapy may be of value in treating untreatable metastatic cancer patients.

Based on the in vitro results obtained in this project, several hypotheses are made with reference to the inhibitory actions of melatonin on human cancers such as the example described above. First, methoxytryptamine, one of the derivatives of melatonin, were found to be the most potent among the tested indoles in inhibiting incorporation of ^{3}H-thymidine in most tumor cell lines. Administration of melatonin either orally or intramuscularly could lead to an increase in the plasma level of melatonin, which could in turn increase the plasma level of methoxytryptamine. The inhibitory action of melatonin in cancer may be a result of an elevation of methoxytryptamine level. However, limited availability of assays to measure the

serum levels of methoxytryptamine and other pineal indoles in patients after melatonin therapy hinders further investigation.

Second, the mitogenic responses of splenocytes to Con A, which is a T cell mitogen, and LPS, which is a B cell mitogen, were found to be the highest in mice receiving melatonin in the drinking water. Splenocytes from the melatonin - treated group produced higher levels of γ-interferon and interleukin-2 than splenocytes from the methoxytryptamine - treated group which in turn produced higher levels of γ-interferon and interleukin-2 than splenocytes from the control group. γ-Interferon is one of the macrophage activating factors. Interleukin-2, known as T cell growth factor, is required for cytotoxic T cell growth. Crude lymphokines from splenocytes of the melatonin - treated group were able to stimulate macrophages of all three groups to produce the highest level of nitrite which is essential to the tumoricidal activity of macrophages. It is consistent with the finding that crude lymphokines from splenocytes of the melatonin - treated group were able to stimulate macrophages of all three groups to achieve the greatest inhibition of the growth of the murine mastocytoma P815 cells. Interestingly, crude lymphokines from splenocytes of the methoxytryptamine - treated group were able to stimulate higher nitrite production when compared to the control group, but lower than the melatonin-treated group by macrophages from the melatonin-treated group and the methoxytryptamine-treated group but not the control group. However, it did not lead to greater inhibition of the growth of murine mastocytoma P815 cells when compared to the control group. Thus the data suggest that melatonin is more potent than methoxytryptamine in stimulating the lymphocytes. On the whole, melatonin was able to enhance the cell-

mediated immune response of the host through an activation of lymphocytes, which include T lymphocytes and B lymphocytes. Maestroni (1986) has demonstrated that melatonin was able to enhance the antibody response in vivo. Macrophages from both melatonin-treated mice and methoxytryptamine-treated mice only responded to activation by crude lymphokines from melatonin - treated mice to achieve a significantly higher inhibition of the growth of murine mastocytoma P815 cells than the control. Methoxytryptamine was less effective than melatonin in enhancing the responses of lymphocytes according to the results obtained in the present investigation. Elucidation of the detailed mechanisms requires further investigations.

Thirdly, only macrophage-mediated cytotoxicity, which was also only restricted to antibody-independent manner, was studied although effector cells of cell-mediated immunity included other cell types such as cytotoxic T cells, natural killer cells, and granulocytes. Macrophages from the melatonin - treated group were found to achieve a higher production of nitrite, superoxide, and tumor necrosis factor-α than the control group. Macrophages from the methoxytryptamine - treated group were found to achieve a lower production of hydrogen peroxide, superoxide but a higher production of tumor necrosis factor-α than the control group. These parameters are related to the tumoricidal activity of macrophages. When in vitro lymphokine activation was not involved, melatonin was found to be more effective than methoxytryptamine in enhancing these functions of macrophages. However, macrophages from the methoxytryptamine - treated group demonstrated a higher nitrite production than macrophages from the melatonin - treated group or the control

group in response to crude lymphokines from any one of the three groups were used. On the whole, methoxytryptamine seemed to be more effective in enhancing the macrophage functions tested than melatonin. This awaits confirmation. If verified, it would interestingly demonstrate that the pineal indoles (melatonin and methoxytryptamine) have slightly different role to play in the immunomodulatory process, that is melatonin stimulates the lymphocytes more potently while methoxytryptamine is more stimulatory to the macrophages.

All of these results show that pineal indoles have antitumor potential. They express it not only in a direct manner, but also in an indirect manner. They have immunoenhancing potential although much more work needs to be done to extend the present findings.

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