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Melatonin modulates allergic lung inflammation

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Abstract: Asthma is an inflammatory lung disease characterized by cell migration, bronchoconstriction and hyperresponsiveness, and can be induced, as an experimental model, by ovalbumin sensitization followed by a challenge. In addition to the well-known immunostimulatory effects of melatonin, research has identified some of its anti-inflammatory properties. In this study, we evaluated the influence of pinealectomy and melatonin administration on cell migration in an experimental model of allergic airway inflammation. We evaluated, in pinealectomized rats treated or not with melatonin, cell migration into the bronchoalveolar fluid, the number of cells and their proliferative activity in the bone marrow, and plasma corticosterone levels. Pinealectomy reduces, 24 hr after the challenge, the total cell number count in the lung and bone marrow cell proliferation, without changing the number of cells in the bone marrow or in the peripheral blood. This fact suggests that melatonin is important in the control of cell recruitment from the bone marrow and the migration of those cells to the lung. Melatonin administration to pinealectomized rats seems to restore the ability of cells to migrate from the bone marrow to the bronchoalveolar fluid. So, the development of specific inhibitors of melatonin would benefit patients with asthma.

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Introduction

Asthma is an inflammatory lung disease characterized by cell migration, bronchoconstriction and hyperresponsiveness [Sheffer, 1991]. Several reports indicate that the antigen activated mast cell releases mediators, such as tumor-necrosis-factor (TNF), arachidonic acid metabolites, proteases, nitric oxide, histamine and 5-hydroxytryptamine, which contribute to cellular influx into the lung [He et al., 1997; Coward et al., 1998]. This is mainly due to the ability of injured epithelia to rapidly recruit circulating inflammatory cells [Coward et al., 1998; Wright et al., 1999]. In fact, the number of lung inflammatory cells including neutrophils, eosinophils and lymphocytes is increased in bronchoalveolar lavage (BAL) obtained from asthmatic patients [Metcalfe et al., 1997]. The subsequent tissue damage leading to airway hyperactivity is a result of the action of toxic cellular agents produced by multiple activated leukocyte populations [Kraneveld et al., 1997].

During the last decade much attention has centered on melatonin (5-methoxy-*N*-acetyltryptamine), one of the secretory products of the diffuse neuroendocrine system. A considerable amount of evidence has demonstrated that melatonin enhances immune function, both at central and at peripheral levels [Pierpaoli and Maestroni, 1987; Guerrero and Reiter, 1992; Maestroni and Conti, 1993]. Melatonin has been shown to enhance interleukin-2 (IL-2) production, T-helper cell activity and also to exert a modulatory effect on lymphocyte proliferation [Gala, 1991]. Indirectly, the increased IL-2 production and T-helper cell activity leads to increased antibody production in response to melatonin [Caroleo et al., 1992].

Melatonin also regulates natural killer (NK) cell cytotoxicity [Hellstrand and Hermodsson, 1987, 1990], antibody production [Jackson et al., 1985; Maestroni et al., 1987a,b], lymphocyte interferon-gamma (IFN-γ) production [Arzt et al., 1988] and T-helper-2 function [Calvo et al., 1995]. Some experiments have also demonstrated an indirect effect of melatonin on the immune system, through the endogenous opioid system, which controls the functions of macrophages and lymphocytes [Maestroni and Conti, 1991; Panerai et al., 1991].

The indoleamine also restores the decrease in lymphocyte proliferation rate and hydrogen peroxide production by macrophages induced by pinealectomy [Martins Jr et al., 1998].

In addition to melatonin immunostimulatory effects, some research has identified its anti-inflammatory properties. Melatonin treatment reduces cell migration and pleural exudation in response to carrageenan [Cuzzocrea et al., 1999a,b] and zymosan [Constantino et al., 1998; Cuzzocrea et al., 1998]. These models, however, consist of non-allergic inflammatory reactions, different from that induced by ovalbumin sensitization followed by challenge, as used herein, which induces a classic allergic-inflammatory reaction.

Taking into account the wide spectrum of biological activities of melatonin and especially its effect on inflammatory cells, as well as the fact that it is produced by mast cells and airways epithelial cells [Kvetnoy, 1999], and considering the importance of the inflammatory response to the development of asthma, we have evaluated the influence of pinealectomy and melatonin administration on cell migration in an experimental model of allergic airway inflammation.

Material and methods

Animals

Male Wistar rats, weighing 160–180 g obtained from the Animal Breeding Unit at the Institute of Biomedical Sciences, were used. The animals were fed ad libitum with commercial chow (Nuvilab, Brazil) and kept in a temperature-controlled room, under a 12:12 hr light/dark cycle (lights on at 07:00 hr). The Ethical Committee on Animal Research at the Institute of Biomedical Sciences, University of São Paulo, approved the experimental procedure.

Induction of experimental allergic asthma

The rats were sensitized with a single intraperitoneal (i.p.) injection of ovalbumin (OA) (10 μg per animal) mixed with aluminum hydroxide (10 mg per animal) as adjuvant. A booster injection was performed subcutaneously at day 7 (2 mL of the OA, 0.1%). Fourteen days after the first sensitization, the rats were exposed, for 15 min to an aerosol of OA (1%) using an ultrasonic inhalator. Non-sensitized rats exposed to a physiological salt solution (0.9% NaCl in distilled water) were used as controls. The period between the inoculation and the challenge was chosen based on the work of Coleman et al. [1983], who demonstrated that the circulating levels of immunoglobulin E (IgE) antibodies increase rapidly, 7–14 days after the i.p. injection of OA.

Pinealectomy

The animals were anesthetized with pentobarbital (45 mg/kg bw) and submitted to pinealectomy using the method of Hoffman and Reiter [1965]. In brief, the anesthetized animals were placed in a stereotaxic apparatus for small animals and a sagittal opening was made on the scalp. The skin and muscles were pushed aside to expose the lambdoidal suture. By means of a circular drill, a disc-shaped opening was made around the lambda and the disc-shaped piece of bone was delicately removed. Thereafter, the pineal gland (which is located just below the posterior venous sinus confluence) was removed with a fine forceps. After a brief hemostasis, the disc-shaped bone was replaced and the scalp was sutured with cotton thread.

Plasma corticosterone levels

Peripheral blood was collected from anesthetized rats (chloral hydrate 400 mg/kg, i.p.) 3 hr after the period of lights off, into plastic syringes containing EDTA (8% in phosphate-saline buffer) from the abdominal aorta. The samples were centrifuged (650g, 10 min) and plasma corticosterone concentration was measured by radioimmunoassay (Amersham's Biotrak).

Plasma IgE levels

Peripheral blood was collected from anesthetized rats, as previously described, and plasma IgE concentration was measured by an enzymatic assay (ELISA, Quibasa Química Básica, MG, Brazil).

Production of TNF-α and INF-γ from peripheral blood mononuclear cells

Peripheral blood was collected from anesthetized rats, as previously described, in heparinized tubes. The samples were centrifuged against a ficoll-hypaque gradient (Hystopaque® 1077) and the mononuclear cells separated. The cells were cultured in RPMI-1640 medium for 48 hr at 37°C in an artificially humidified atmosphere of 5% CO_2 in air at sterile conditions, in the presence of phytohemagglutinin (1 μg/mL) [Kenyon et al., 2000]. Cultures were performed in a LAB-LINE Microprocessor CO_2 incubator (LAB LINE) in 96-well plates (Corning, NY), 1×10^6 cells per well

(total volume, 200 μL). TNF and INF production were measured by using ELISA kits (Rat BD Opt EIA 2696 and 2697, USA).

Blood leukocyte counts

Warmed conscious rats were held in small boxes and the blood was collected from the tail vein using a heparinized plastic syringe. Samples were diluted (1:20) in Turk fluid (3% acetic acid in water) for the counting of the total cell number in a Neubauer chamber. Differential analysis was performed in blood smears stained by the Rosenfeld method.

Collection and analysis of BAL fluid

The rats were killed with chloral hydrate (> 400 mg/kg i.p.). After exsanguination by abdominal aorta incision, a plastic cannula was inserted into the trachea and the BAL performed using sodium phosphate buffer (PBS) (20 mL) according to Tavares de Lima et al. [1998]. The BAL fluid was centrifuged at 170g for 10 min and then resuspended in 1 mL of PBS. Aliquots of the samples were diluted (1:20) in 0.2% crystal violet (dissolved in 30% acetic acid) for the assessment of total cell number in the Neubauer chamber. For differential analysis, 70 μL of each sample was employed in Citospin for the attainment of slides. The slides were stained by the Rosenfeld method.

Collection and analysis of bone marrow fluid

A femur fragment from the exsanguined rat was removed and flushed with PBS (5 mL). The bone marrow fluid was centrifuged at 170g for 10 min and then resuspended in 1 mL of PBS. Aliquots of the samples were diluted (1:20) in 0.2% crystal violet (dissolved in 30% acetic acid) for total cell count assessment in a Neubauer chamber.

Incorporation of [2-^{14}C]thymidine into cultured bone marrow cells

The rats were killed with chloral hydrate (> 400 mg/kg i.p.). After exsanguination (abdominal aorta), a femur fragment was removed and flushed with PBS (5 mL). The bone marrow fluid was homogenized and centrifuged at 170g for 10 min and then resuspended in RPMI-1640 medium.

The bone marrow cells were cultured in RPMI-1640 medium for 48 hr at 37°C in an artificially humidified atmosphere of 5% CO_2 in air at sterile conditions. Cultures were performed in a LAB LINE Microprocessor CO_2 incubator (LAB LINE) in 96-well plates (Corning, NY), 1×10^6 cells per well (total volume, 200 μL). After 48 hr in culture, more than 98% of cells were viable, as measured by trypan blue dye exclusion. The cells were then pulsed with 20 μL of 0.02 μCi [2-^{14}C]thymidine (sp. act. 56.0 mCi/nmol), diluted in sterile PBS, yielding a final concentration of 1 μg/mL. The cells were then maintained under these conditions for additional 16 hr and automatically harvested by a multiple cell harvester and filter papers cat. no. 11731 (Skatron Combi, Suffolk, UK). The paper discs containing labeled cells were counted in 1 mL of Ecolume™ scintillation cocktail, in a Beckman-LS 5000TD liquid scintillation (Beckman Instruments, Fullerton, CA).

Experimental groups and statistical analysis

The animals were divided in four groups: (1) control group (CONTROL): intact rats that were not submitted to the sensitization process and, consequently were not challenged. The animals were submitted to the inhalation of saline aerosol 24 hr before sacrifice (n = 9); (2) intact-sensitized group (INTACT): intact rats that were sensitized to OA and challenged 14 days latter (n = 10); (3) pinealectomized-sensitized group (PINX): rats were pinealectomized 30 days before the sensitizations process to OA. The challenge with OA aerosol was done 14 days after the immunization (n = 10); (4) melatonin-treated pinealectomized-sensitized group (PINX Mel): same as the PINX group except for the fact that the animals received a daily i.p. injection of 0.3 mg of melatonin starting 15 days after surgery and ending 24 hr after the challenge procedure (n = 12).

Considering the day of the immunization to OA as day zero, pinealectomy was done 30 days earlier; melatonin administration started 15 days before and ended 15 days after immunization with OA; the booster subcutaneous injection was done on day 7 and the challenge was done on day 14. All animals were sacrificed on day 15.

Statistical analysis

The data are expressed as mean ± standard error of the mean (S.E.M.) and were analyzed using the statistical procedure of parametric analysis of variance followed by the Bonferroni's multiple comparison test using GraphPad Prism 3.0 for Windows NT (GraphPad Software Inc., San Diego CA).

Results

The total number of cells present in the BAL fluid from intact-sensitized and challenged rats was

significantly increased when compared with the CONTROL group. When the same challenge was imposed to the pinealectomized and sensitized rats, the total number of cells present in the BAL fluid was significantly reduced ($P < 0.001$) compared to the INTACT group, and was not different from the CONTROL group values (Fig. 1A). The daily treatment of pinealectomized animals with melatonin (0.3 mg/day), for 1 month starting 15 days after surgery, restored the pulmonary inflammatory response, as the number of cells found in the lung fluid 24 hr after the challenge with OA was higher than that found in the PINX group ($P = 0.001$) and was not statistically different from that of the INTACT group (Fig. 1A).

The number of eosinophils, neutrophils, mononuclear cells and lymphocytes in the BAL presented the same pattern of changes as those observed in Fig. 1A (data not shown).

To better evaluate the effect of pinealectomy on cell recruitment, both blood and bone marrow cells from rats were quantified. The data are presented in Fig. 1B,C, respectively. The data indicate that neither sensitization nor pinealectomy affected leukocyte counts in the peripheral circulation. The total cell number in bone marrow, however, was reduced in OA-sensitized and

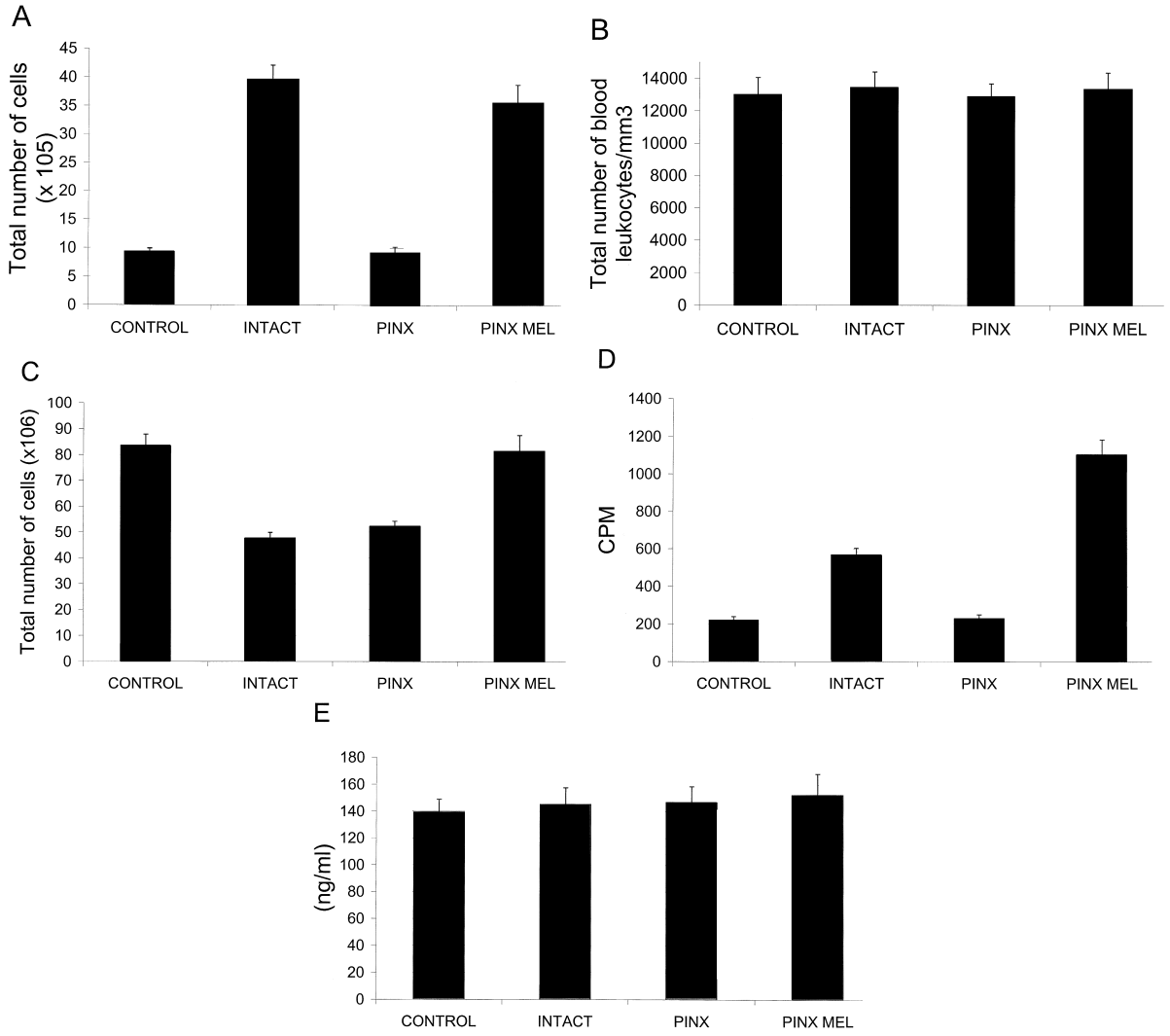

Fig. 1. BAL total cell number (**A**), number of leukocytes/mm^3 of blood (**B**), total number of bone marrow cells (**C**); bone marrow cell proliferation rate (**D**) and plasma corticosterone concentration (**E**) measured in no stimulated rats (CONTROL, n = 9), immunized and challenged rats (INTACT, n = 10), pinealectomized-immunized and challenged rats (PINX, n = 10) and pinealectomized-immunized and challenged rats that were treated chronically with 0.3 mg melatonin daily (PINX-MEL, n = 12) The comparison between groups was done using ANOVA followed by the Bonferroni's multiple comparison test. (**A**) *($P < 0.05$) statistically different from CONTROL and PINX; **($P < 0.01$) statistically different from PINX and CONTROL. (**C**) *($P < 0.05$) and **($P < 0.01$) statistically different from INTACT and PINX. (**D**) *($P < 0.05$) statistically different from CONTROL and PINX; **($P < 0.01$) statistically different from CONTROL, INTACT and PINX.

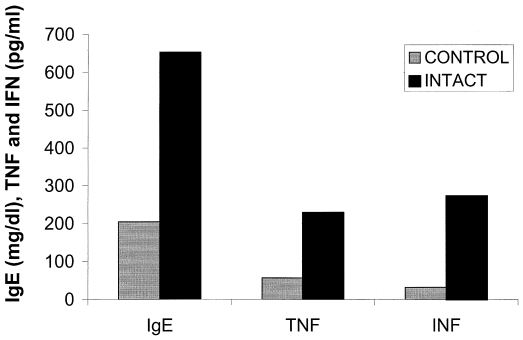

Fig. 2. Concentration of plasma immunoglobulin E (IgE) and that of TNF-α and INF-γ produced by peripheral blood mononuclear cells cultivated for 48 hr, obtained from no stimulated rats (CONTROL, n = 9), immunized and challenged rats (INTACT, n = 10), pinealectomized-immunized and challenged rats (PINX, n = 10) and pinealectomized-immunized and challenged rats that were treated chronically with 0.3 mg melatonin daily (PINX-MEL, n = 12) The comparison between groups was done using Student's *t*-test. $^*P < 0.05$ for comparison with CONTROL group.

challenged animals ($P < 0.01$) (Fig. 1C). PINX rats still showed a reduction in the total number of cells in bone marrow 24 hr after the challenge ($P < 0.05$). Melatonin replacement to PINX rats augmented the number of cells in bone marrow to a count similar to that observed for the CONTROL group (Fig. 1C).

Bone marrow cells obtained from intact-sensitized rats 24 hr after they were challenged by OA showed a higher rate of cell proliferation when compared with CONTROL animals ($P < 0.05$) (Fig. 1D). Animals subjected to PINX and then sensitized and challenged showed a bone marrow cell proliferation rate significantly smaller than that of the INTACT rats ($P < 0.01$), and identical to the rats that were not sensitized (CONTROL) (Fig. 1D). Treatment of pinealectomized challenged rats with melatonin, however, augmented (12-fold) the proliferative response of such cells (Fig. 1D).

Corticosterone levels, as assessed by RIA, 24 hr after the OA challenge, indicated that neither the antigen challenge, pinealectomy nor melatonin treatment influenced plasma corticosterone concentrations when compared with that obtained from the CONTROL group (Fig. 1E).

OA challenge increased plasma IgE concentration ($P < 0.01$) in control rats, as well as the production of TNF-α and INF-γ by peripheral blood mononuclear cells cultured for 48 hr ($P < 0.01$, Fig. 2).

Discussion

Allergic asthma is a multifactorial disease, characterized by bronchial hyperresponsiveness, the presence of IgE antibodies to inhalant allergens and often by enhanced total serum IgE levels, and by genetic and environmental factors. Experimentally, a similar feature is obtained in animals using OA sensitization, followed by a challenge with low particle mass concentrations of aerolized OA [De Sanctis et al., 1999]. The immunological and inflammatory aspects of our model were confirmed by the increase in plasma IgE concentration and in that of TNF-α and INF-γ production by cultured mononuclear cells obtained from OA-sensitized animals (Fig. 2). The changes in plasma IgE, TNF and INF concentrations confirm the efficiency of our protocol in inducing an experimental feature of allergic asthma [Renzi et al., 1996; Dery and Bissonette, 1999] and are in agreement with those reported by Kenyon et al. [2000].

In this model of experimental allergic asthma, pinealectomy prevented cell migration to the BAL, as induced by the OA challenge. The chronic treatment of pinealectomized rats with melatonin, given daily restored the ability of inflammatory cells to migrate to the lung when the animal was challenged by aerolized OA.

It is well known that melatonin can influence some aspects of the immune response such as antibody production [Kuribayashi et al., 1981; Caroleo et al., 1992], including the production of IgG1 isotype in response to an OA challenge, which increased in the presence of melatonin [Shaji et al., 1998]. IgG1 antibodies are of particular interest, as they can bind to mast cell receptors and mediate cell degranulation [Nielsen et al., 1994; Miyajima et al., 1997]. Considering the effect of melatonin upon immunoglobulin production, we evaluated whether the reduced production of immunoglobulins in sensitized, pinealectomized rats were involved in the reduction of cell migration through a decrease of mast cell degranulation. Unpublished observations of our laboratory show that mast cell degranulation, as evaluated by the measurement of the contraction of a tracheal slice from previously immunized rats in the presence of OA [Schultz, 1910; Dale, 1913], seems to be normal for both groups (INTACT and PINX), indicating that there was no change in immunoglobulin production in response to pinealectomy. Confirming our findings related to the absence of alterations in immunoglobulin production, we did not find changes on plasma IgE concentration, they remained the same in the three groups that were immunized (653.2 ± 32.1,

674.1 ± 40.3 and 663.1 ± 51.8 mg/dL for INTACT, PINX and PINX MEL, respectively, and 204.5 ± 22.4 mg/dL for the control group). Our data reinforce that, at least the model used here, mast cell function during allergic experimental asthma is not altered by melatonin.

Eosinophils, neutrophils, mononuclear cells or lymphocytes in the BAL presented the same pattern of changes as those observed for the total number of BAL cells, indicating that their migration to the inflammatory site is modulated by melatonin, and is reduced in pinealectomized rats. These results are in agreement with those of Mathison et al. [1994], who showed reduced neutrophil and alveolar macrophage function and migration in the late-phase pulmonary inflammatory response to challenged rats infected with the nematode *Nippostrongylus brasiliensis*, after decentralization of the superior cervical ganglia. It is important to emphasize that this surgical procedure abolishes the sympathetic innervation to the pineal gland, inducing a functional pinealectomy, and a loss of circulating melatonin [Zigmond et al., 1985; Bartol et al., 1997].

To better understand the mechanisms underlying the decrease in cell number in the BAL obtained from pinealectomized rats we counted blood cell and bone marrow cell number, and also determined the proliferative activity of cultivated bone marrow cells. The number of peripheral blood leukocytes did not change either in response to pinealectomy or to melatonin treatment in pinealectomized rats.

The total number of cells in the bone marrow 24 hr after the challenge with OA was not altered by pinealectomy. In fact, the challenged rats presented a reduction in total cell number, which was the same as that of pinealectomized rats. After melatonin administration to pinealectomized rats, however, an increase in total cell number in bone marrow, which became similar to values found in the CONTROL group was found. This was probably because in these animals the proliferation rate of bone marrow cells was 12-fold higher than in the PINX group.

The change in the number of BAL cells induced by pinealectomy, taken together with the changes observed in the number of cells in the bone marrow and in its proliferative responses, indicated an effect of melatonin in modulating cell migration in two different sites, from the bone marrow cavity to the blood and from the blood to the bronchoalveolar fluid. The mechanisms involved, however, remains to be clarified.

Considering the anti-inflammatory effect of corticosterone and the large body of data regarding the effects of melatonin and pinealectomy on plasma corticosterone concentration [Touitou, 1989], we evaluated the concentration of this hormone in the plasma. We did not observe any changes in that parameter, indicating that in our model of experimental allergic-inflammatory asthma, plasma corticosterone, evaluated 24 hr after challenging and 3 hr after lights off, was not a factor in cell migration to the lung.

Pinealectomy reduces, 24 hr after the challenge, the total cell number count in the lung, and bone marrow cell proliferation, without changing the number of cells in the bone marrow or in the peripheral blood. This fact suggests that melatonin is important to control cell recruitment from the bone marrow and the migration of those cells to the lung. Melatonin reposition in pinealectomized rats seems to recover the ability of cells to migrate from the bone marrow and to the BAL. So, the development of specific inhibitors of melatonin would benefit patients with asthma.

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