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# Melatonin Increases the Intensity of Respiratory Burst and Prevents L-Selectin Shedding in Human Neutrophils *in Vitro*

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**Effects of melatonin priming of neutrophils and subsequent increase of phorbol 12-miristate 13-acetate stimulated respiratory burst were investigated on the modulation of L-selectin shedding and MAC-1 upregulation. Respiratory burst related $H_2O_2$ production and adhesion molecule expression were quantified by flow cytometry. Phorbol 12-miristate 13-acetate dose dependence of intracellular oxidation and adhesion molecule expression showed no relationship between respiratory burst intensity and MAC-1 expression or L-selectin shedding. Treatment of cells with 12.5 nM phorbol 12-miristate 13-acetate resulted in less than 20% of the respiratory burst response, however it induced 91.7% of total MAC-1 expression and 62.8% of L-selectin shedding. Melatonin priming experiments showed also no connection between the extent of respiratory burst and MAC-1 expression, however melatonin priming almost completely prevented L-selectin down-regulation elicited by phorbol 12-miristate 13-acetate, without affecting MAC-1 expression. It is suggested that melatonin may inhibit metalloproteases responsible for L-selectin cleavage.** 

The pineal gland and its indole hormone, melatonin, have been shown to be capable of modulating immune function. Indeed, the administration of a pineal extract or melatonin produces thymic hyperplasia (1), increases the antibody response (2), natural killer cell activity (3), the proliferative response to Con A (4, 5), and antigen presentation by macrophages (6); it prevents age-related thymus involution (7). In addition, melatonin activates human monocytes and induces interleukin-1 secretion, reactive oxygen intermediates, and cytotoxicity against tumour cells by a pathway involving protein kinase C (8). Previous results from our laboratory (9) have shown that melatonin was also able to markedly increase the extent of respiratory burst (RB) induced by phorbol 12-miristate 13-acetate (PMA) in human neutrophils, suggesting a potential role of the indole to strengthen the host defense against the bacterial and fungal pathogens. However, activation of neutrophils must be limited to those cells at the inflammatory site, so that damage to normal tissues is minimized. Thus, it is of importance to evaluate whether melatonin may influence the events leading to extravasation of neutrophils into tissues, which is the cellular hallmark of acute inflammation (10). This process is dependent on the coordinated regulation of the expression and function of at least two families of cell adhesion molecules in neutrophils (11): leukocyte adhesion molecule L-selectin, and the $\beta_2$-integrins (MAC-1 or CD11b/CD18) (12-14).

The goal of this study was to determine the effects of melatonin on the expression of integrin (MAC-1) and L-selectin (CD62L) adhesion molecules on the surface of human neutrophils. In addition, the existence of an eventual relationship between the intensity of RB and the expression of the adhesion molecules was also evaluated.

## MATERIALS AND METHODS

Blood samples were obtained by venipuncture from the authors and the technical staff of the Cytology Center after overnight fasting, and collected into Vacutainer tubes with heparin as anticoagulant (Becton Dickinson, Rutherford, NJ).

Dihydrorhodamine 123 (DHR), Molecular Probes, Eugene, Oregon, was dissolved in DMSO at a concentration of 30 mM and stored in 25 $\mu$l aliquots at −70° C. Phorbol 12-myristate 13-acetate, Sigma Chemical Co., was dissolved in DMSO to give 1.6 mM stock solution and stored in 10 $\mu$l aliquots at −70° C. To obtain the working solution (10 $\mu$M), PMA was diluted in Dulbecco's PBS, then further in the cell suspension to 100 nM final concentration. Melatonin stock solution (1 mM) was freshly prepared in PBS, then further diluted to the final working solutions in Dulbecco's PBS.

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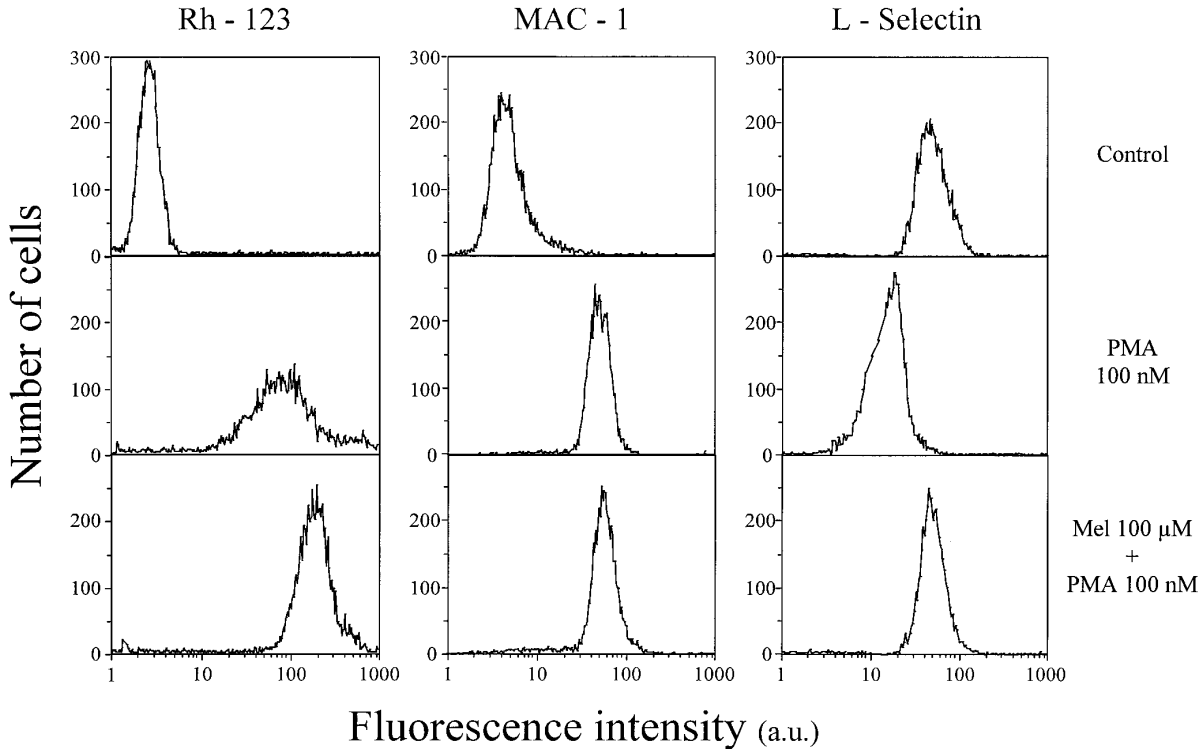


**FIG. 1.** Respiratory burst and expression of MAC-1 and L-selectin in neutrophils upon PMA activation with or without melatonin priming. Neutrophils were pre-incubated with or without 100 $\mu$M melatonin for 10 min. at 37° C, before the addition of 100 nM PMA for an additional 30 min. Intracellular $H_2O_2$ production (Rh-123 fluorescence), MAC-1 and L-selectin expression were determined by flow cytometry. Histograms show profiles of log fluorescence intensity. Representatives of minimum seven separate experiments are shown in each case.

*Isolation of leukocytes.* To avoid spontaneous oxidative burst activity of neutrophils due to the isolation procedures, blood was depleted of erythrocytes by 1 gr. sedimentation on top of Ficoll avoiding, this way, any contact of leukocytes with the separation media **(**15). The supernatant leukocyte-rich plasma was aliquoted and used for the analysis of RB and adhesion molecules expression.

*Flow cytometric assay of RB.* The intracellular production of reactive oxygen species, including $H_2O_2$, in PMA stimulated neutrophils was quantified in individual cells by flow cytometry using a procedure, based on the oxidation of non-fluorescent DHR to the green fluorescent rhodamine 123 (Rh-123) (16).

Cells ($1 \times 10^6$ cells/ml) were suspended in a microfuge tube containing Dulbecco's PBS plus 5 mM D-glucose and 1 $\mu$M DHR. The suspension was incubated for 5 min. at 37° C before the addition of PMA. The conversion of DHR to its fluorescent derivative, Rh-123, was assessed 30 min. after PMA stimulation, incubating the cells at 37 °C. In samples used for monitoring the effect of melatonin, cells were treated with different concentrations of the hormone for 10 min. at 37 °C before incubating them with DHR.

Neutrophil-associated fluorescence was analyzed using a Coulter Epics V flow cytometer (Coulter, Hialeah, Fl). An electronic gate was drawn around granulocytes on the basis of forward and right angle scatter characteristics. The argon laser was tuned to 488 nm and the green fluorescence of Rh-123 was detected between 500 and 540 nm. Data were analyzed by program packages provided by the manufacturers. On every occasion average intensity of fluorescence emitted by at least 10,000 cells was measured.

*Flow cytometric assay of MAC-1 and L-selectin expression.* Mouse anti-leu 15 antibody directed against the $\alpha$-chain (CD11b) of the CD11b/CD18 adhesion receptor complex and mouse anti-leu-8 antibody directed against L-selectin, both obtained from Becton Dickinson, Heidelberg, Germany, were used in the fluorimetric studies. Fluorescein isothiocianate conjugated anti-mouse $IgG_{2a}$ (Pharmigen, San Diego, USA) was used as secondary antibody. After stimulation of neutrophils for 30 min. with 100 nM PMA, preceded or not by a 10 min. melatonin treatment, the cells were fixed with 2% paraformaldehyde in PBS. They were then incubated with the appropriate monoclonal antibody at 4° C for 30 min., washed extensively, and incubated with the secondary antibody (diluted in PBS + 5% FCS) for another 30 min. The samples were centrifuged, washed and resuspended in PBS, and stored in the dark at 4° C until the fluorometric analysis. Negative controls were obtained by omitting the first antibodies. In some experiments isotype matched control antibodies were used to evaluate non-specific binding.

## RESULTS

Before evaluating the effect of melatonin on the adhesion molecule expression, the dose-response curve of the neutrophil RB of each individual taking part in the study was analyzed with the use of different PMA concentrations (from 10 to 150 nM, data not shown). As expected (9, 17) great variability in the neutrophil response to PMA was observed among the subjects considered. At 100 nM PMA a sustained response was achieved in all samples, therefore this dose was chosen for subsequent experiments. In agreement with our previous results (9), 100 $\mu$M melatonin has been initially used to prime the cells, since this concentration

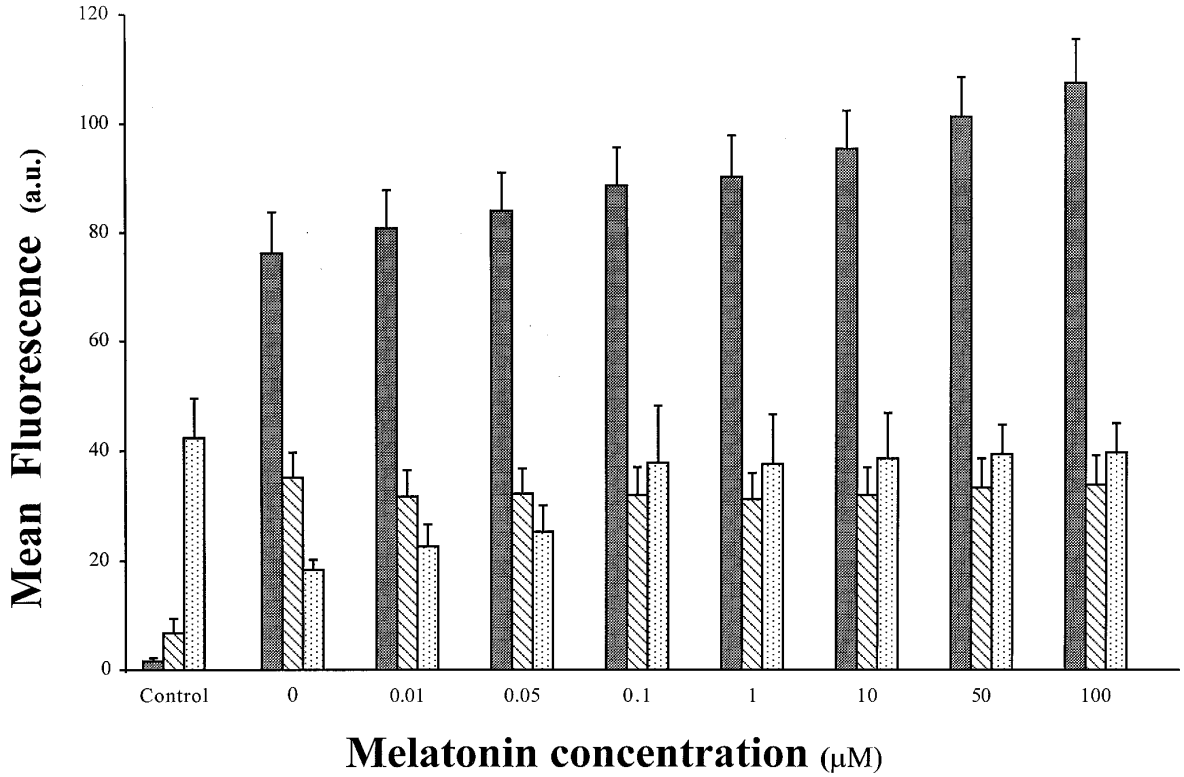


**FIG. 2.** Effect of melatonin concentration on PMA-stimulated respiratory burst (Rh-123) ■, as well as on MAC-1 ▨, and L-selectin ▩ cell surface expression. Neutrophils were pre-treated with selected concentrations of melatonin in the concentration range of 100 $\mu$M-10 nM for 10 min. at 37 °C and then stimulated with 100 nM PMA for 30 min at 37 °C. The values shown are mean ± SEM of seven separate experiments. Control = neutrophils treated with Dulbecco's PBS buffer alone. 0 = neutrofils treated with 100 nM PMA only.

was able to increase the RB induced by PMA. Representative examples of the histograms obtained measuring the Rh-123 fluorescence as well as the expression of MAC-1 and L-selectin are shown in Fig. 1. As expected, stimulation with 100 nM PMA for 30 min. resulted in a sustained increase of Rh-123 fluorescence, indicating that RB was activated, as well as in an increase of MAC-1 and a reduction of L-selectin expression. When neutrophils were primed by incubation for 10 min. with 100 $\mu$M melatonin, then stimulated with PMA, a sustained increase of RB occurred. However, the expression of MAC-1 remained unchanged, whereas L-selectin shedding was significantly decreased. It has to be noted that treatment of the cells with melatonin alone did not induce RB and did not modify adhesion molecule expression (data not shown).

Although these findings clearly support that melatonin considerably influenced the RB, as well as the shedding of L-selectin, it must be noted that the experiments were performed using pharmacological doses of the hormone. Dose response experiments were performed with melatonin concentrations ranging between 100 $\mu$M and 10 nM in order to test whether the same responses occur with hormone concentrations approaching the physiological level. The results from five separate experiments are shown in Fig. 2. The increment of RB elicited by melatonin decreased in parallel with the decrease of hormone concentration, whereas the expression of MAC-1 remained unchanged. It is of interest to note that an almost complete block of L-selectin shedding was obtained with melatonin concentrations as low as 100 nM. Physiological doses of the hormone (50 and 10 nM) resulted in a lower, but significant, inhibition of shedding.

It is also suggested that these two events are not quantitatively correlated, because the modulation of RB did not result in a modulation of adhesion molecules expression (Fig. 1). In order to verify this hypothesis, the intensity of RB was modulated stimulating the neutrophils with different concentrations of PMA (from 6.25 to 100 nM), and analyzing Rh-123 fluorescence as well as MAC-1 and L-selectin expression. As shown in Fig. 3, the treatment of the cells with doses of PMA as low as 12.5 nM resulted in less than 20% of the RB response, but was able to induce 91.7% of MAC-1 expression and 62.8% of L-selectin shedding. Considering the lowest concentration of PMA used (6.25 nM), it was observed that together with RB practically absent, 67.6% of MAC-1 was expressed whereas more than 27% of L-selectin was shedded. These data confirm the non-correlated behavior of RB intensity, L-selectin shedding and MAC-1 up-regulation.

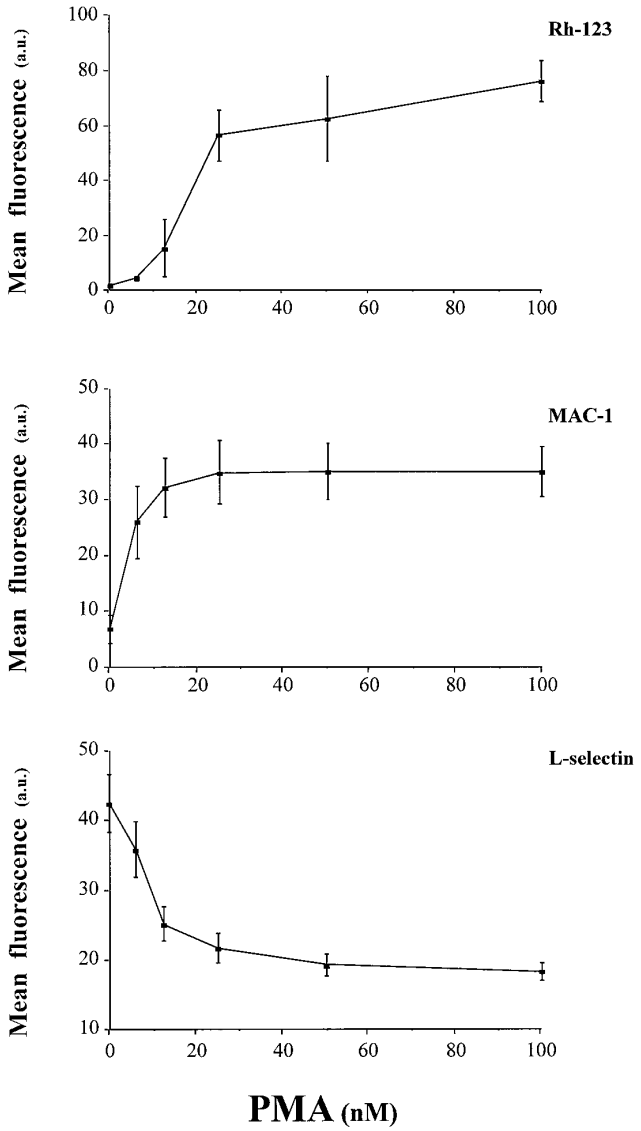


**FIG. 3.** Effect of PMA on the respiratory burst (Rh-123), MAC-1 and L-selectin cell surface expression in neutrofils. Cells were treated with different concentrations of PMA for 30 min. at 37 °C. The values shown are mean ± SEM of seven separate experiments.

## DISCUSSION

Leukocyte extravasation is essential in the inflammatory response. This process can be divided into three steps: initial interaction of leukocytes with activated endothelium (rolling), leukocyte activation with firm adhesion to endothelial cells and, finally, their extravasation into the surrounding tissues (18). Several adhesion molecules are involved in the process of adhesion and migration of leukocytes through vascular endothelium at sites of inflammation (12-14). Among them, two classes of surface antigens have been shown to play a major role in neutrophil-endothelial cell interactions: L-selectin and $\beta_2$-integrins. The L-selectin molecule (also known as LAM-1, LECAM-1, LEU-8) is constitutively expressed and functional on inactivated leukocytes. L-selectin has a key role in the initial attachment of circulating neutrophils to endothelium. This molecule is responsible for the rolling of leukocytes along the vascular walls, as a prerequisite to arrest movement and firm adhesion, thus allowing the interaction of other molecules (integrins), which account for trans-endothelial migration and cell extravasation to the target tissues (14). Stimulation of neutrophils with activating or chemoattractant factors, such as PMA, C5a, FMLP, TNF-$\alpha$, LTB4, LPS, and IL-1 causes a rapid decrease in the number of surface L-selectin molecules.

$\beta_2$-integrins (CD11a/CD18 or LFA-1, CD11b/CD18 or MAC-1, and CD11c/CD18 or p150, 90) are essential in the neutrophil extravasation process. Among them, MAC-1 molecules are the most important in neutrophil adherence and extravasation, in vivo, with no effect on cell rolling. A low number of MAC-1 molecules are expressed on inactivated neutrophil surface, but they are up-regulated, and became functionally active upon neutrophil stimulation (19-20).

Apart the activating and chemoattractant factors listed before, adherence of neutrophils to vascular endothelial cells seems also to be stimulated by oxygen derived free radicals. Indeed, a link between the generation of oxidant species and leukocyte recruitment has been demonstrated in ischemic-reperfused tissues, were superoxide dismutase, and the xantine oxidase inhibitor, allopurinol, reduced leukocyte infiltration (21). In a recent paper, it has been reported that different biologically occurring oxidative molecules and oxygen free radicals caused up-regulation of MAC-1 and L-selectin shedding in human neutrophils, in vitro (22). In these experiments, the peroxidative stress was induced adding $H_2O_2$ to the bathing medium, and the concentrations of the oxidant able to change the expression of the adhesion molecules were rather high (from 0.1 to 10 mM). In the present paper, the production of oxygen derived species by human neutrophils was modulated by priming the cells with melatonin, a pineal hormone that is able to increase the PMA activated RB (9). In addition, the extent of RB was modulated by stimulating the cells with different doses of PMA. The results from these experiments (Figs. 2, 3) clearly show that there is no direct relationship between oxygen-derived free radical production by PMA and adhesion molecules expression in human neutrophils.

The results of the experiments performed when priming the cells with melatonin have particular merit, namely melatonin had an unexpected effect on the PMA caused adhesion molecule expression even if neutrophils had been stimulated to produce more $H_2O_2$, because it did not influence the expression of MAC-1, but completely prevented L-selectin shedding. The

mechanism through which melatonin avoids L-selectin shedding is at present unknown. L-selectin shedding occurs via enzymatic cleavage at a membrane proximal site, thereby releasing the extracellular domain (23, 24). The enzyme involved is a metalloprotease (25), thus, it can be hypothesized that melatonin may directly or indirectly inhibit this enzyme. From a physiological point of view, the present finding supports that melatonin has proinflammatory properties. This conclusion is based on the results of recent reports showing that metalloprotease inhibitors block L-selectin down-regulation from the cell surface of stimulated neutrophils, reduce their rolling velocity under hydrodynamic flow, resulting in increased neutrophil accumulation (26). In addition, it has been shown that, among other effects, nonsteroidal antiinflammatory drugs strongly inhibited the L-selectin-mediated interaction of neutrophils with activated endothelial cells by inducing rapid cleavage and shedding of the membrane L-selectins (27).

In conclusion, results of the present work demonstrate that the extent of RB, the expression of MAC-1 and L-selectin shedding are not quantitatively correlated. Moreover, it has been shown that melatonin is a powerful inhibitor of L-selectin shedding.

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