

Letter

Diurnal variation of CD63 expression on activated blood basophils: a pilot study

Basophils contribute to immediate-type hypersensitivity reactions and to different diseases. Basophils are highly granulated leukocytes and can release their mediators in response to high-affinity IgE receptor crosslinking.¹ The activation marker CD63 is present in secretory vesicles and expressed on the basophil cell surface after activation.² The basophil activation test is a tool in clinical allergology to quantify in vitro activated basophils by flow cytometry.³

Mammals have developed an endogenous circadian clock. The circadian system is involved in many physiologic processes.⁴ Exacerbation and manifestation of allergic diseases appear more frequently between midnight and morning and show prominent 24-hour variations, indicating an involvement of the circadian clock in allergy.⁵ Based on findings on the involvement of the circadian clock in effector cells, including mast cells and eosinophils, and, hence, the release of their mediators,⁶ we investigated diurnal variation of basophil activation. To avoid the influence of specific allergens on basophil activation, we analyzed basophils of volunteers without allergy in a pilot study.

Fourteen 22- to 55-year-old subjects without atopy at the Dia-konhøjskolen (Aarhus, Denmark) agreed to donate their blood and were recruited to the study, which was conducted from late April to early May 2014. Each volunteer completed an allergy questionnaire used in the allergy clinic at Aarhus University Hospital. Volunteers had regular sleep and wake patterns, took no medications, did not report allergic symptoms, and did not show elevated total or specific IgE serum levels against the ImmunoCap (Thermo Fisher Scientific, Waltham, Massachusetts) inhalation allergens timothy grass, cultivated rye, birch pollen, mugwort pollen, *Cladosporium herbarum*, house dust mite, and cat and dog dander. To study variation of basophil activation dependent on time of day, 4 mL of heparinized blood and serum was drawn at 4 PM and at 4 AM the following morning. Melatonin was measured in serum by enzyme-linked immunosorbent assay (IBL International GmbH, Hamburg, Germany) to confirm the circadian rhythm. Serum melatonin levels were significantly elevated at 4 AM (median 99 pg/mL, interquartile range 129–75) compared with levels at 4 PM (median 8 pg/mL, interquartile range 13–6; $n = 14$, $P < .0001$), confirming the circadian rhythm of the volunteers. Permission to conduct the study was obtained from the ethics committee of Aarhus County (J Nr 1-10-72-76-14).

One hundred microliters of heparinized blood was incubated with 0.5 pg/mL to 50 μ g/mL of antihuman IgE (BD Pharmingen, San

Jose, California) at log intervals and with fluorescein isothiocyanate antihuman CD63 and Alexa Fluor 647 antihuman CD193 (CCR3; BioLegend, London, United Kingdom) at 37°C for 30 minutes. Phosphate buffered saline (Thermo Scientific, Oxoid, Hampshire, United Kingdom) was used as a negative control that did not induce CD63 expression on the basophil cell surface. Blood was lysed by the addition of 2 mL of FACS Lysing Solution (BD Biosciences) for 8 minutes, washed, and resuspended in phosphate buffered saline. Analysis of expression levels of the basophil activation marker CD63 was performed using a BD FACS Canto II flow cytometer. Basophils were selected based on low granularity and CD193 positivity (SSC^{low}/CD193^{high}). CD193 was chosen as a primary basophil marker expressed on basophils, eosinophils, and a subset of T cells.⁷ The percentage of CD63⁺ basophils and the mean fluorescence intensity of CD63 was identified by FACS Diva software. Isotype controls had been used during the validation of basophil activation test. Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad, San Diego, California). Dose–response curves were fitted to a 4-parameter nonlinear regression model. Data are presented as mean $\pm$ SEM of 14 subjects. Two-tailed t test for paired samples was used to test for differences between CD63⁺ cells and CD63 mean fluorescence intensity, respectively, at 4 PM and 4 AM. The significance level was set at $P < .05$.

In this pilot study, we found that basophil sensitivity of volunteers without allergy showed diurnal changes in response to IgE-dependent activation. Figure 1 shows a significant decrease in basophil sensitivity at 4 AM ($n = 14$, $P = .0298$) when assessed by the mean fluorescence intensity of CD63⁺ basophils, suggesting

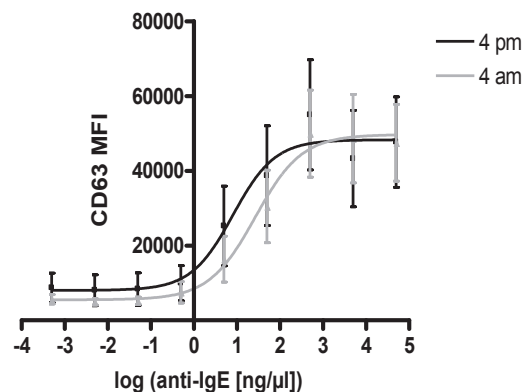


Figure 1. Comparison of sensitivity of human basophils at 4 AM and 4 PM. The mean fluorescence intensity (MFI) of CD63 was plotted against the increasing concentration of antihuman IgE. Basophils were significantly more sensitive at 4 PM than at 4 AM ($n = 14$, $P = .0298$).

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that basophils of persons without allergy are less sensitive early in the morning. Notably, we did not find diurnal variation of reactivity assessed by the more commonly used percentage of CD63⁺ cells in response to anti-IgE at 4 AM and 4 PM ($P = .607$, data not shown). Hence, our data support the finding that the mean fluorescence intensity is an important parameter in basophil activation test.

Mast cells and eosinophils, main effector cells of allergic reactions aside from basophils, recently have been found to exhibit a functional clock mechanism. Clock genes are responsible for generating the circadian rhythm and the clock has been found to control activation of mast cells and eosinophils, resulting in circadian production and release of their mediators.⁶ These findings assume that the circadian clock influences and controls allergic reactions and not vice versa. Moreover, blood basophil numbers display circadian variations.⁸ In addition, we found that the intensity of the activation marker CD63 expressed on basophils exhibits diurnal variation. Very recently, daily variations in basophil reactivity have been reported in patients with seasonal allergic rhinitis, with an increase in CD203c⁺ basophils at 7 AM compared with 7 PM.⁹ However, we analyzed the circadian rhythm of the sensitivity of basophils of volunteers without allergy. Because donor selection, time points, activation markers, and readout differed between the previous and present studies, the results are not directly comparable. Certainly, further experiments and measurements of more than 2 time points will be necessary to clarify this issue and to explain the higher frequency of allergic symptoms between midnight and morning. At activation, expression of CD63 correlates with degranulation and the release of histamine and with the severity of allergic reactions.^{2,10} Thus, basophil activation, mediator release, and clock gene expression around the clock and the correlation of circadian allergic symptoms of patients should be analyzed in a follow-up study.

In conclusion, this pilot study provides a first hint that the circadian clock might regulate the intensity of basophil response in IgE-dependent allergic reactions.

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