ORIGINAL RESEARCH PAPER

Seasonal changes in activities of human neutrophils in vitro

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Abstract

Objective and design We present a retrospective analysis of previously collected blood samples to determine whether the immune response of neutrophils depends on the season i.e., short versus long days, in which blood samples were collected.

Methods The bactericidal activity and adhesive capacity of neutrophils, the production of reactive oxygen species (ROS), and CD11b/CD18 molecule expression level were investigated. The investigated neutrophils were divided into two groups based on the time of blood collection: the winter season with short days and the summer season with long days.

Results We found seasonal variation in measurements of all the analyzed functional responses of neutrophils to stimuli. The strongest adhesion, as well as maximum values of ROS production, was presented by neutrophils isolated from the summer group. The highest bactericidal activity of neutrophils was also observed in blood donors from summer group.

Conclusions The magnitude of the immune functional activity of neutrophils varies with the season of the year and is decreased in winter.

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Introduction

Numerous mechanisms have been proposed to explain seasonal susceptibility of the host to infection. One of them may involve the modulation of the immune system's response caused by the annual light/dark cycle, also called photoperiod, which is mediated by melatonin [1, 2]. Some experimental data on animal and human models provide evidence that the functional activity of immune cells varies in conjunction with the photoperiod [3–7].

Neutrophils are an important element of host innate immunity and its ability to destroy pathogens is critical to defense against infection. During infection, polymorphonuclear neutrophils are engaged in the eradication of invading pathogens by phagocytosis, which is preceded by adhesion to the vascular endothelium (e.g., via $\beta2$ integrin CD11b/CD18), migration in extravascular tissue, and finally ingestion and degradation of pathogens via oxygen-dependent reactive oxygen species (ROS) and oxygen-independent mechanisms [8–12].

We have gathered data through a series of experiments over a period of 10 years. Analysis has showed a considerable variation in the functional response of neutrophils to exogenous stimuli in vitro. The noticeable heterogeneity of the parameters examined was in part the result of differences between blood sample donors and in part due to the time of blood collection. Depending on the season in which the blood samples were collected, we observed small or great stimulation, no effect at all or even suppression of the neutrophil activity in vitro. The great variations in

functional responses of neutrophil and lack of consistency made interpretation and statistical analysis of results very difficult [13–18]. Bronson [19] believes that some humans are seasonally photoresponsive while others are not, and that individual variation may be the cause of the inconsistencies in cell reactivity measurements.

In this article we present an analysis of previously collected data to determine whether the immune activity of neutrophils truly depends on the season and to assess potential seasonal changes in their biological functions in relation to the effect of periodic environmental factors like the seasonal length of daylight. To examine this hypothesis, a series of measures was constructed for each season and the results were then analyzed statistically.

The measures we analyzed were: the adhesive capacity of neutrophils, the expression level of CD11b/CD18, and the capacity of neutrophils for ROS production and killing of *Escherichia coli* bacteria.

Materials and methods

Study protocol

This investigation included 155 healthy male subjects who had been donors of neutrophils in our laboratory since the year 2000. These individuals did not suffer from inflammatory diseases or other illness and did not take any medications. The individuals were divided into two groups: winter and summer season, according to whether the blood samples had been collected on short or long days. The winter group included individuals whose blood sample was taken between November and March and the summer group between April and October. No samples were collected in July and August.

Chemical reagents

Polymorphprep was obtained from Axis Shield (Oslo, Norway). Hanks' balanced salt solution (HBSS), phorbol 12-myristate 13-acetate (PMA), formyl-methionyl-leucyl-phenylalanine (fMLP), tumor necrosis factor-alpha (TNF-α) human recombinant, 3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyltetrazolium bromide (MTT) and saponin were purchased from Sigma Aldrich (St. Louis, MO, USA). Mouse IgG2a anti-human CD11b PE-conjugated antibodies clone D12 and BD CellFIX solution were purchased from BD Biosciences (San Jose, CA, USA). Luminol was obtained from Serva (Heidelberg, Germany). McFarland Standard was purchased from bioMerieux (Marcy l'Etoile, France). Fetal bovine serum (FBS) was obtained from Gibco Invitrogen Cell Culture (Inchinan, Scotland). RPMI 1640 medium and phosphate buffered saline (PBS) were purchased from Biomed (Lublin, Poland).

Isolation of neutrophils

Heparinized venous blood samples were layered onto Polymorphprep and centrifuged at $500 \times g$ for 30 min at room temperature. The top band containing mononuclear cells was removed while the lower one, containing polymorphonuclear cells, was collected. The neutrophils were washed twice with PBS. Cell viability (>95%) and cell purity (>95%) were assessed by Trypan blue exclusion and May–Grünwald–Giemsa staining, respectively. Finally, neutrophils were resuspended in HBSS or RPMI 1640 culture medium as indicated.

Bacteria

The *E. coli* LI1 strain was obtained from the collection of the Institute of Microbiology and Immunology, University of Lodz, Poland. The bacteria were grown in a tryptic soy agar for 18 h at 37°C, then harvested and washed 3 times with PBS by centrifugation (2,500$\times g$, 15 min, +4°C). The number of bacteria was adjusted spectrophotometrically according to the McFarland Standard (550 nm, ELISA reader Multiscan RC, Labsystem, Finland) to 1.5×10^9 cells/ml. The bacteria (1×10^8/ml) were opsonized with 20% pooled human serum in RPMI 1640 medium (20 min, 37°C, with gentle agitation). Afterwards, bacteria were washed once with RPMI 1640 medium.

Adhesion assay

Neutrophil adherence to a plastic surface is a simple quantitative technique for examining the ability of human neutrophils for active adherence in vitro. Briefly, neutrophils (1×10^6 cells/well in RPMI 1640 medium with 10% of FBS) were distributed into 24-well Nunclon plates (Nunc, Roskilde, Denmark). Then, 1 µg/ml of PMA, 1 µM of fMLP and 20 ng/ml of TNF-α, respectively, were added to neutrophils or cells were left untreated. Adhesion was performed for 60 min at 37°C in a 5% CO_2 atmosphere. Non-adherent cells were removed by careful washing of the surface of the wells with PBS and adherent neutrophils were exposed to MTT solution (2 mg/ml PBS) containing 10 ng/ml of PMA for 60 min. Formazan produced by adherent neutrophils was dissolved in isopropanol. The absorbance was measured at 595 nm and 630 nm dual wavelengths in the ELISA reader. The absorbance of formazan is directly related to the number of adherent neutrophils. The data were expressed as optical density (OD).

Measurement of expression of CD11b/CD18 surface molecules

The expression of CD11b/CD18 molecules on the surface of neutrophils was detected using the flow cytometry method. Freshly isolated neutrophils (2×10^6 cells/ml in HBSS) were stimulated with 1 µM of fMLP or left untreated for 20 min at 37°C in a 5% CO_2 atmosphere. Placing all samples on ice stopped the reaction, and then cells were stained with mouse IgG2a antihuman CD11b PE-conjugated antibodies for 30 min, at 4°C, in the dark. Thereafter, all samples were fixed in BD CellFIX solution (500 µl) and stored at 4°C until analyzed on a Beckman Coulter (USA) flow cytometer equipped with Cytomics FC 500 MPL System with MXP Software for cell acquisition and data analysis. The neutrophils were gated on the forward and side scatter. The results were expressed as median fluorescence intensity (MFI), which correlates with the expression of surface antigen.

Production of ROS by neutrophils

The production of ROS was assessed using the luminol enhanced chemiluminescence (CL) method. CL was measured in a Fluoroscan Ascent FL fluorometer (Labsystem). Neutrophils were distributed into 96-well white plates (1×10^5 cells/well in HBSS). Subsequently, 10 µM of luminol and 1 µg/ml of PMA or 1 µM of fMLP were added to cells. The CL reading was recorded for 30 min at 2-min intervals. The CL intensity was given in relative light units (RLU). The data were expressed as the area under the curve of CL versus time (RLU total).

Bactericidal activity of neutrophils

The MTT colorimetric microassay was used for measuring the bactericidal activity of neutrophils. Neutrophils (5×10^5 cells/well in RPMI 1640 medium with 5% of FBS) and *E. coli* (5×10^6 cells/well in RPMI 1640 medium with 5% of FBS) were added to 96-well plates [OD sample]. The control samples contained: (i) the neutrophils in culture medium [OD 90% killing], or (ii) bacteria in the culture medium [OD 0% killing]. The reaction mixtures were incubated for 60 min at 37°C in a 5% CO_2 atmosphere. Afterwards, neutrophils were lysed with 0.05% of saponin for 20 min at room temperature. MTT solution (0.5 mg/ml) was then added to the bacteria for 60 min at 37°C. The living bacteria converted the yellow tetrazolium salt of MTT to the dark blue formazan product. Formazan was solubilized with isopropanol. Absorbance (OD) was measured at 595 and 630 nm dual wavelengths in the ELISA reader. The absorbance of formazan is directly related to the number of viable bacteria. The error threshold was calculated as 10% and obtained values were subjected to a 10% discount. The percentage of bacteria killed by neutrophils was calculated by the formula: [1 − (OD sample − OD 90% killing)/(OD 0% killing − OD 90% killing)] × 90%.

Statistical analysis

Results are presented as means ± SD. Statistical analysis was performed with the nonparametric Mann–Whitney U test using Statistica 8.0 for Windows. Statistical significance was defined as $p < 0.05$.

Results

We found seasonal variation in the measurements of all analyzed functional responses of neutrophils to stimuli. The magnitude of the adhesive activity measurements of the summer group neutrophils in response to TNF-α or PMA stimulation was larger in comparison with that of the winter group. The strongest adhesion was shown by neutrophils isolated from individuals in April and May
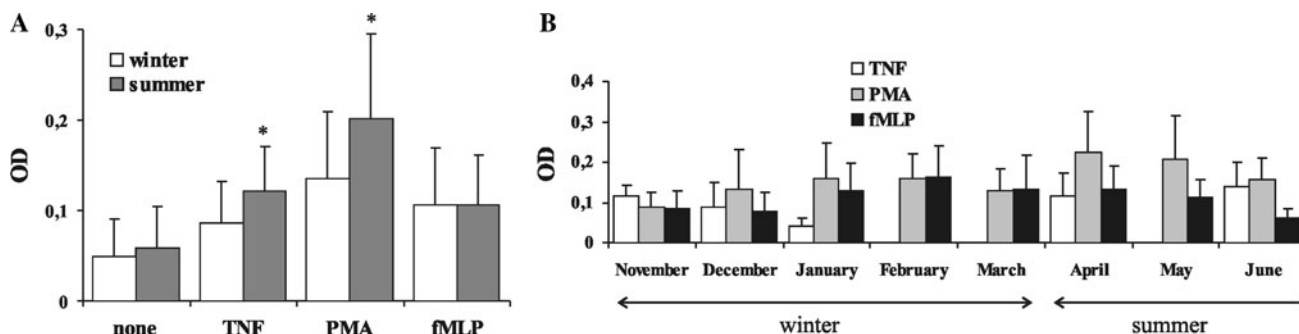
Fig. 1 The adhesive activity of neutrophils isolated from healthy individuals of summer and winter groups. Neutrophils were incubated with PMA, fMLP and TNF-α or left untreated for 60 min. Adhesion was estimated using MTT assay. Data are expressed as means of optical density (OD) ± SD. **a** Adhesive activity of neutrophils during summer and winter. *Asterisk* summer group ($n = 32$) versus winter group ($n = 31$), $p \leq 0.02$, Mann–Whitney U test. **b** Adhesive activity of neutrophils in different months
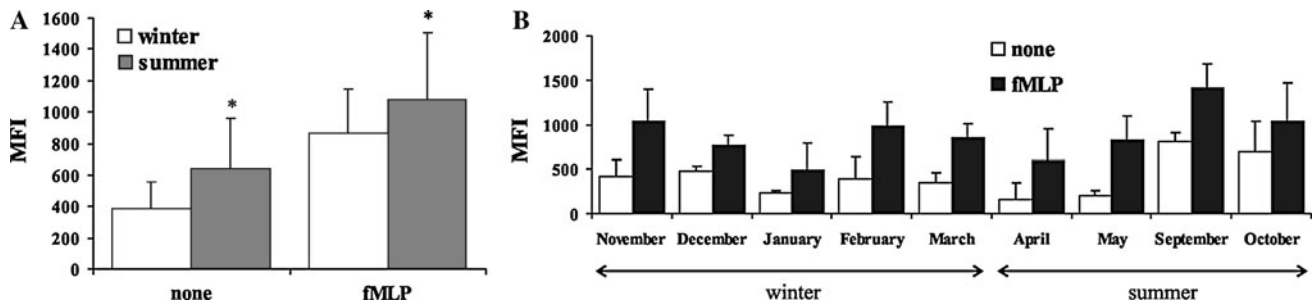
Fig. 2 Expression of CD11b/CD18 molecules on neutrophils isolated from individuals of summer and winter groups. Neutrophils were stimulated with fMLP or left untreated for 20 min. Expression of CD11b/CD18 was estimated using flow cytometry method. Data are expressed as median fluorescence intensities (MFI) ± SD. **a** Expression of CD11b/18 on neutrophils during summer and winter. *Asterisk* summer group ($n = 21$) versus winter group ($n = 32$), $p \leq 0.04$, Mann–Whitney U test. **b** Expression of CD11b/CD18 on neutrophils in different months

(Fig. 1a, b). However, it is noteworthy that no seasonally dependent differences were observed in the neutrophil adhesive response to fMLP stimulation (Fig. 1a). The analysis of CD11b/CD18 expression as a function of the annual light/dark cycles is presented in Fig. 2. We found that unstimulated and fMLP-stimulated neutrophils of the summer group demonstrated significantly higher CD11b/CD18 expression level than those of the winter group (Fig. 2a). The most intense expression of this molecule was observed on neutrophils isolated in September and October (Fig. 2b). As shown in Fig. 3a, the ability of the summer group neutrophils to produce ROS in response to stimulation with PMA or fMLP was significantly greater than that of the winter group. The maximum values of ROS production were observed in the neutrophils obtained from individuals in April and May (Fig. 3b).

ROS production in neutrophils and CD11b molecule expression level coincided with the ability of neutrophils to kill bacteria. Neutrophils of the summer group demonstrated a significantly stronger capacity to kill *E. coli* bacteria in comparison to neutrophils of the winter group (Fig. 4a). The highest bactericidal activity of neutrophils was observed in June and September (Fig. 4b).

Discussion

This review and the analysis of our laboratory results from the last 10 years demonstrated the seasonal variation in the measurement of functional activities of human peripheral blood neutrophils [17]. We found that in both the unstimulated and in vitro fMLP-stimulated neutrophils the adhesive abilities of neutrophils, ROS production, and CD11b/CD18 expression levels were increased in the samples collected during long days versus short days, resulting in intensified bactericidal properties of the summer group neutrophils.

Seasonal variation in the activity of the immune system of some mammals is well documented. It has been described that phagocytosis and ROS production by neutrophils and monocytes of the Siberian hamster on short days were significantly lower than on long days. The proliferation of splenic lymphocytes of hamsters exposed to short days was also suppressed. On the other hand, cytotoxic activity of natural killer (NK) cells on short days was enhanced compared with their activity on long days [3, 4, 20]. There are limited and contradictory data on the activity of human immune cells in correlation with the annual light and dark cycles. Killestein et al. [21] demonstrated that
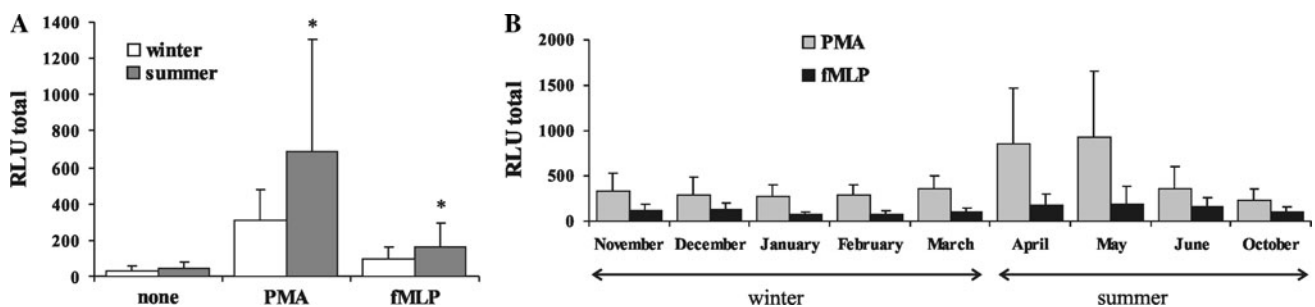
Fig. 3 ROS production by neutrophils isolated from individuals of summer and winter groups. Neutrophils were stimulated with PMA and fMLP or left untreated. The ROS production was determined using chemiluminescence assay. Data are expressed as means of relative light units (RLU) total ± SD. **a** ROS production by neutrophils during summer and winter. *Asterisk* summer group ($n = 71$) versus winter group ($n = 84$), $p \leq 0.0009$, Mann–Whitney U test. **b** ROS production by neutrophils in different months
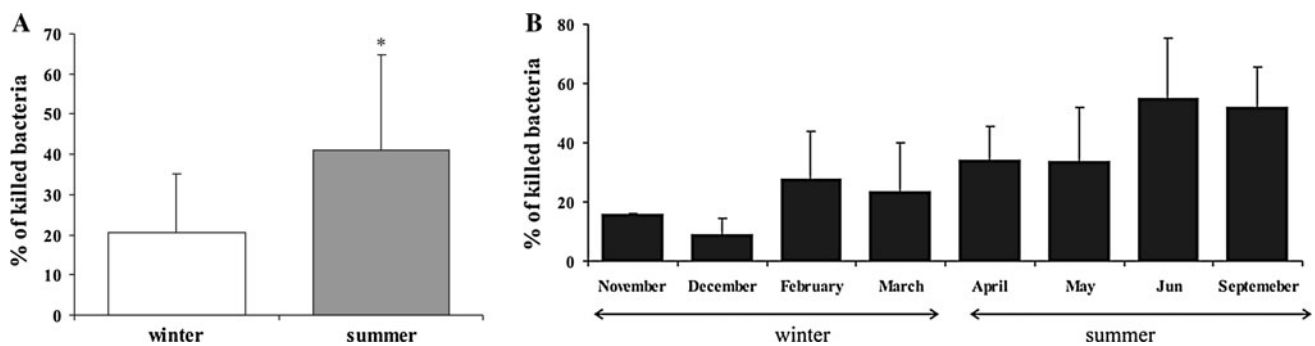
Fig. 4 Bactericidal activity of neutrophils isolated from individuals of summer and winter groups. Neutrophils were incubated with *E. coli* bacteria for 60 min. The viability of bacteria was determined by MTT assay. Data are expressed as optical density (OD) ± SD.

a Bactericidal activity of neutrophils during summer and winter. *Asterisk* summer group (n = 25) versus winter group (n = 21), $p \leq 0.003$, Mann–Whitney *U* test. b Bactericidal activity of neutrophils n different months

maximum values of TNF-α production by stimulated peripheral blood mononuclear cells were obtained when cells were isolated from individuals' blood in the autumn. In contrast, Myrianthefs et al. [22] showed, when testing the whole blood, that the production of TNF-α during September was significantly lower than that in February and March.

The seasonal rhythm of host susceptibility to particular pathogens (bacterial and viral) and infectious diseases is an interesting subject for many researchers. It was demonstrated that incidence of influenza peaks at different times in different countries. In the USA the peak is observed from November to March, in the UK from December to April, and in Japan from December to March [1, 2, 23]. As mentioned, the seasonal susceptibility of mammalian species, including humans, to infection depends on the prevalence and/or virulence of a pathogen, human behavior, and environmental changes. The major signal responsible for seasonal changes connected with the daily photoperiod is mediated by melatonin. Melatonin is secreted by the pineal gland; its synthesis and secretion occurs at night and is inhibited by light. The duration of its release is proportional to the length of the night [2, 23]. The relationship between melatonin level and the activity of human immune system cells has been well documented [5, 24, 25]. It has been demonstrated that melatonin modulated cytokine production by leukocytes, the proliferation and differentiation of lymphocytes, and the activity of NK cells. The effect of melatonin on immune cells is dependent on the specific membrane receptors present on leukocytes [26–28]. It is worth mentioning that there are other physiological sources of melatonin in humans, e.g., lymphocytes. However, the regulation of melatonin synthesis outside of the pineal gland is poorly characterized [29, 30].

In our study, we decided to forgo the test of human neutrophil responsiveness to stimuli after exposure to melatonin in vitro, since other investigators have already tested some of the functions of phagocytes in the presence of this hormone. Pieri et al. [31] reported that a low dose of melatonin increased ROS production by human neutrophils, while a high dose (2 mM) significantly decreased ROS production by PMA-stimulated neutrophils. Silva et al. [32] demonstrated that melatonin inhibited HOCl production in neutrophils and decreased their ability to kill *Staphylococcus aureus* bacteria.

The aim of this study was to document that variations in measurements of neutrophil response in vitro are not caused by measurement error but by cyclical, seasonal changes. The results confirms that immune functional activity of neutrophils, including killing bacteria in vitro, varies with the seasonality of the year and is decreased in winter. This seasonal responsiveness and reactivity of neutrophils may be due to the annual light and dark differences. Our results therefore demonstrate that a seasonal variation in innate immune system function is feasible. Additionally, our work supports other researchers' studies mentioned above. Knowledge of seasonal rhythms in neutrophil functional reactivity is clinically important for the appropriate interpretation of laboratory results. It should also be taken into account in the long term in experiments in vitro and in vivo, as well as in planned immunotherapeutic strategies for chronic, infectious diseases.

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