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Seasonal influence on mitogen and cyclosporin responses of peripheral blood lymphocytes

Fotios V. Michelis *, Andreas K. Delitheos, Ekaterini Tiligada

Department of Pharmacology, Medical School, University of Athens, M. Asias 75, GR-11527 Athens, Greece

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ABSTRACT

The immune response and lymphocyte activation in particular are affected by environmental factors. In vivo and in vitro experiments demonstrate variability in lymphocyte activation according to seasonal changes. This study focused on the effects of season on the ex vivo mitogen-induced activation of lymphocytes from peripheral blood of healthy humans living in a temperate climate, as well as the ex vivo lymphocyte activation of rabbits living under constant laboratory conditions. The possible impact of season on the action of the immunosuppressant drug cyclosporin A (CsA) on lymphocyte activation was investigated in both species. Cultured peripheral blood lymphocytes from human donors (n = 13, 22–63 years of age) and from animals housed under 12:12 hour light:dark cycle were stimulated with phytohemagglutinin (PHA) in the absence or presence of 10 and 25 μg/mL CsA. Lymphocyte activation was assessed by morphometric analysis under a light microscope.

Percentages of unactivated lymphocytes, activated lymphoblasts and aberrant cells reflecting cytotoxicity were determined. Human lymphocytes demonstrated a significant decrease in response to PHA during the winter months, in comparison to the rest of the year. In contrast, the peripheral blood lymphocytes of rabbits housed under constant conditions did not demonstrate similar variations in response to PHA stimulation. The immunosuppressive action of cyclosporin A on this experimental model was unaffected by the observed seasonal variation in mitogen response in humans. These findings may guide research towards the identification of factors associated with the seasonality of the immune response and its potential influence on therapeutic interventions.

1. Introduction

Ample evidence indicates that seasonality drives numerous physiologic changes and pathologic conditions in mammalian species, including humans, which may affect the evaluation of medical treatment or the course of disease [1–3]. It is becoming increasingly evident that the immune response of vertebrates is subjected to various environmental factors, a phenomenon which is frequently reflected in the seasonal variation of the response [2,4]. Without disregarding the discrepancies arising from interspecies differences and the diversity of methodological approaches employed in both clinical settings and research protocols [5,6], variations have been demonstrated for a range of immune parameters in animals and humans. For instance, reports argue for circannual differences in the mitotic activity of mitogen-activated human blood lymphocytes [7], photoperiodic effects on T cell-dependent humoral immunity in hamsters [8] and seasonal changes in tumor necrosis factor production in the macrophages of ground squirrels [9].

Annual variations in photoperiod are considered to play an important role in the seasonal changes of numerous aspects of immune function [10]. A number of studies have demonstrated that immune system activation is compromised during the short days of winter [3,7,8,11], although many of the studies have produced controversial results [5,12]. Moreover, environment-driven changes in host physiology may possibly underlie, at least in part, the seasonal cycles of infectious diseases in different parts of the world [4]. Despite the fact that mounting data suggest that changes in photoperiod and the melatonin pulse may influence both cellular and humoral immunity [2], seasonal immunological changes are not as well characterized for humans as for other mammals.

This study focused on the investigation of ex vivo mitogen-induced activation of lymphocytes from peripheral blood of healthy human individuals living in the temperate climate zone and the comparison of lymphocyte activation between the different seasons. Peripheral blood lymphocyte (PBL) activation was also determined with the same methodology for rabbits living under constant controlled laboratory conditions including steady photoperiod and temperature. Additionally, the possibility of a seasonal effect on the action of the immunosuppressant drug cyclosporin A (CsA) on PBL activation in vitro was investigated.

* Corresponding author. Tel.: +30 2107462575; fax: +30 2107462554.
E-mail address: fotismichelis@hotmail.com (F.V. Michelis).

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2. Materials and methods

All aspects of the study were conducted in accordance with the approved published ethics guidelines and with the approval of the national and institutional review board. Written informed consent was obtained from all human participants. All animal studies were performed by fully trained and experienced personnel and complied with ethical codes and regulations.

2.1. Media, reagents and drugs

The culture medium RPMI-1640, containing 20 mM HEPES, 10% (v/v) fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine, and the T-cell mitogen phytohemagglutinin (PHA) were purchased from Biochrom KG (Berlin, Germany). The commercially available preparations of heparin (Heparin®, Leo) and cyclosporin A (CsA, Sandimmun®, Novartis) were used. Giemsa was obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Human subjects and blood sampling

Heparinized (100 IU/mL heparin) peripheral whole blood samples were collected bimonthly during 2000–2001, from 13 (5 females, 8 males) healthy, medication-free Greek adult volunteers age 22–63 years (mean ± SD: 33 ± 13 years), at fixed time points (09.00–10.30 h), the morning after an overnight fast and at least 12 h after exercise and were immediately used for culture. Pregnant women were excluded from the study. All donors lived in Athens, Greece and gave informed consent. White blood cell counts were within the normal range in all subjects (data not shown). In total, 71 observations were included in the study. In some analyses, the recruited donors were subdivided into age groups of 20–40 years (mean ± SD: 28.5 ± 5 years, n = 11; 4 women, 7 men) and 60 years (n = 2; 1 woman, 1 man), as well as into non-smokers (n = 8; 5 women, 3 men) and smokers (n = 5; 1 woman, 4 men).

2.3. Laboratory animals and blood sampling

Heparinized (160 IU/mL heparin) blood samples were obtained during 2000–2001 from the auricular artery of 6 male and 3 female New Zealand white rabbits of 2.9–3.3 kg body weight, at fixed time points (09.00–10.30 h), and were used for cell culture immediately. The animals, purchased from an approved commercial breeder, were acclimatized and maintained singly in stainless steel cages under controlled conditions of 12:12 h light:dark cycle, 20 ± 2 °C and 60 ± 5% relative humidity, and they received a standard diet and water ad libitum. In total, 32 observations were included in the study.

2.4. Cell culture and determination of lymphocyte activation

Fresh peripheral blood (0.5 mL) was diluted with 4.5 mL culture medium. Lymphocyte culture and evaluation of activation were performed as described previously [1,13]. Briefly, ex vivo lymphocyte activation was induced by stimulation with 5 μg/mL PHA, and control samples in the absence of PHA were used to determine the extent of spontaneous lymphocyte activation. The effects of CsA on lymphocyte activation were evaluated by incubating human and rabbit lymphocyte cultures in the presence of 10 and 25 μg/mL CsA diluted in RPMI. Since maximal lymphocyte activation with PHA has been documented to occur approximately 48 h after addition of the mitogen [14], incubation was performed for 48 h at 37 °C in humidified atmosphere containing 5% CO_2/95% air. The samples were centrifuged at 500 ×g for 10 min and the precipitate received 7 mL of 75 mM KCl in order to lyse red blood cells.

Aliquots of the fixated with 3:1 methanol:glacial acetic acid lymphocyte suspensions were stained with 4% (w/v) Giemsa and submitted to morphometric analysis under a light microscope (Olympus, Japan) at ×1000 magnification [13,15]. The total number of counted cells in each sample was 300. The percentage of small, deeply basophilic unactivated lymphocytes and larger activated lymphoblasts with less dense nuclei (Fig. 1) was determined. The quantification of aberrant cells was used as a means of identification of cytotoxic activity [1,13]. The absolute lymphocyte numbers in randomly chosen samples of fixated lymphocyte suspensions were counted in a Neubauer hemocytometer.

2.5. Statistical analysis

The percentage of unactivated lymphocytes, lymphoblasts and aberrant cells was determined in each sample in triplicate and the results were expressed as mean ± SD. Significant differences between means were determined using paired samples t-test and ANOVA followed by Scheffé or Dunnett's test, with $p < 0.05$ being regarded as acceptable level of significance. The correlation coefficient (r) was determined by the Spearman's test. Statistical analyses were performed using SPSS for Windows version 19 (SPSS Inc., IL, USA). Seasons were defined as recognized in temperate regions of the northern hemisphere, spring beginning March 21, summer June 21, autumn September 23, and winter December 22.

3. Results

3.1. Mitogen-induced activation of human peripheral blood lymphocytes

The absolute lymphocyte count in the fixated human peripheral blood lymphocyte (*h*PBL) samples was 1431 ± 404 cells/mm^3 and no statistically significant differences were observed ($p > 0.7$, ANOVA) between inter- and intra-individual responses, during the time period the study was performed.

In both unstimulated and mitogen-stimulated *h*PBL cultures, the proportion of activated cells (Fig. 1) was significantly higher than the unactivated or aberrant lymphocytes ($p < 0.001$, ANOVA). Statistically significant increases in lymphoblast proportions were observed in PHA-stimulated *h*PBL cultures compared to the absence of PHA in the respective blood samples ($p < 0.01$, ANOVA). The simultaneous reduction of the proportion of unactivated lymphocytes with respect to spontaneous blastogenesis ($p < 0.01$, ANOVA) validated the ex vivo PHA-induced activation of the cultured *h*PBLs (Fig. 2).

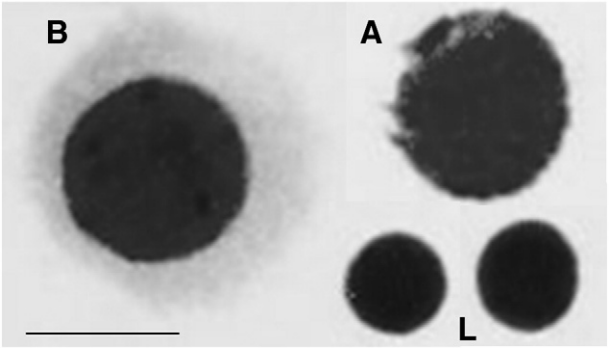

Fig. 1. Microscopic appearance of Giemsa-stained human peripheral blood unactivated lymphocytes (L), activated lymphoblasts (B) and aberrant cells with distorted membrane (A), cultured in the presence of 5 μg/mL phytohemagglutinin. Bar represents 10 μm.

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