

FLOW CYTOMETRIC ASSAY OF RESPIRATORY BURST CAPACITY IN PERIPHERAL BLOOD MONOCYTES OF GAUCHER TYPE 1 PATIENTS

ODREĐIVANJE SPOSOBNOSTI RESPIRATORNOG PRASKA U MONOCITIMA PERIFERNE KRVI OBOLELIH OD GOŠEOVE BOLESTI TIP 1 METODOM PROTOČNE CITOMETRIJE

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Summary

Background: There is an apparently increased tendency toward infections in patients with Gaucher disease, possibly due to defective neutrophil function rather than a decreased neutrophil count. Since macrophages are the main cell type affected in Gaucher disease, our aim was to determine the contribution of these cells to the susceptibility of Gaucher patients to infection by studying the respiratory burst capacity of peripheral blood monocytes.

Methods: The study was performed in eleven Gaucher type 1 patients and eleven sex and age matched control subjects by measuring peripheral blood monocytes' respiratory burst capacity using flow cytometry. The respiratory burst capacity was measured as dihydrorhodamine-123 median fluorescence in patients and respective controls.

Results: There was no statistical difference in the median fluorescence among the patients and respective controls ($p > 0.05$) after phorbol 12-myristate 13-acetate stimulation. Also, statistical difference was not reached among patients treated with enzyme replacement therapy at the time and those untreated.

Conclusions: Flow cytometry might represent a more accurate and more reliable measure of respiratory burst compared to the methods of other researchers. Respiratory burst

Kratka sadržaj

Uvod: Naizgled povećana sklonost infekciji kod bolesnika sa Goševom bolešću verovatno je posledica defektne funkcije neutrofila, a ne smanjenog broja ovih ćelija. Pošto su makrofagi ćelije koje su najviše pogođene u Goševoj bolesti, cilj našeg rada je bio da se odredi doprinos ovih ćelija osetljivosti bolesnika na infekcije, ispitivanjem sposobnosti respiratornog praska u monocitima periferne krvi.

Metode: Ispitivanje je urađeno kod jedanaest bolesnika sa Goševom bolešću tipa 1 i jedanaest kontrolnih ispitanika odgovarajućeg pola i uzrasta, merenjem sposobnosti respiratornog praska monocita periferne krvi metodom protočne citometrije. Respiratorni prasak je određivan merenjem medijane fluorescencije dihidrorodamina-123 kod bolesnika i kontrolnih ispitanika.

Rezultati: Statistički značajna razlika u medijani fluorescencije nije nađena ($p > 0,05$), poređenjem bolesnika i kontrolnih ispitanika, nakon stimulacije forbol-12-miristat-13-acetatom. Takođe, razlika nije nađena ni prilikom poređenja bolesnika lečenih enzimskom supstitucionom terapijom i onih koji nisu dobijali terapiju u momentu ispitivanja.

Zaključak: Protočna citometrija mogla bi da predstavlja precizniju i pouzdaniju meru respiratornog praska u poređenju sa metodima drugih istraživača. Poremećaj respiratornog

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List of abbreviations: ERT, Enzyme replacement therapy; PBS, phosphate-buffered saline; RPMI, Roswell Park Memorial Institute; DHR, dihydrorhodamine; PMA, phorbol 12-myristate 13-acetate; SSI, severity score index; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); DHE, dihydroethidium; EB, ethidium bromide.

disturbance in monocytes does not seem to contribute to increased susceptibility to infection in Gaucher patients.

Keywords: Gaucher disease, respiratory burst, monocytes, flow cytometry, dihydrorhodamine-123

Introduction

Gaucher disease is the most common lipid storage disease caused by lysosomal beta glucocerebrosidase deficiency inherited as an autosomal recessive trait. Undegraded substrate, glucosylceramide, accumulates intracellularly, primarily in cells of mononuclear phagocyte origin. It is now apparent that pathology is caused not just by the burden of glucosylceramide storage, but by macrophage activation which could potentially explain some of the pathological features of the disease such as osteopenia, activation of coagulation, hypermetabolism and gammopathies (1). The disease is usually classified into non-neuronopathic (type 1) and neuronopathic forms (type 2 and 3), the latter much less frequent than the former. Typical clinical presentation of type 1 disease involves hepatosplenomegaly, blood cytopenias and bone lesions. The development of specific treatment, namely enzyme replacement therapy (ERT) led to amelioration of many symptoms and improved quality of life in these patients.

Increased tendency toward infections is reported in patients with Gaucher disease, possibly due to defective neutrophil function rather than decreased neutrophil count (2).

Since macrophages are the main cell type affected in Gaucher disease, our aim was to determine the contribution of these cells to susceptibility of Gaucher patients to infection by studying the respiratory burst capacity of peripheral blood monocytes.

Materials and Methods

Patients and controls

The study was performed in eleven Gaucher type 1 patients and eleven sex and age matched control subjects. The informed consent was signed by the subjects or their parents prior to drawing venous blood. The patients' blood was taken during regular check-up for disease activity markers (chitotriosidase level, ferritin, liver enzymes and acid phosphatase). Control subjects either volunteered or were evaluated for various conditions known not to activate macrophages in an outpatient unit. The study was approved by the ethical committee of the institution.

Flow cytometry assay of peripheral blood monocytes' respiratory burst capacity

Leukocytes were prepared from diluted heparinized venous blood by overlaying on 75% Percoll

praska monocita periferne krvi izgleda da ne doprinosi povećanoj sklonosti infekcijama među bolesnicima sa Gošeovom bolešću.

Cljučne reči: Gošeova bolest, respiratorni prasak, monociti, protočna citometrija, dihidrorodamin-123

gradient and centrifuged at $800 \times g$ for 20 minutes at 20 °C. Cells overlaying Percoll were transferred to a clean tube and washed three times with Mg^{2+} - and Ca^{2+} -free phosphate-buffered saline (PBS; pH 7.2). After third wash, cell pellet was resuspended in RPMI-1640 medium, counted and their viability was determined by trypan blue exclusion and number of cells in suspension was adjusted to 5×10^6 cells/mL.

First, 2.5×10^5 cells were preincubated for 5 minutes with dihydrorhodamine-123 (DHR-123) at final concentration $1 \mu\text{mol/L}$, and then incubated for 30 minutes at 37 °C with or without phorbol 12-myristate 13-acetate (PMA, 100 ng/mL) as the stimulant. Afterwards, samples were immediately placed on ice. Flow cytometry acquisition was performed within 10 minutes.

Monocytes were electronically gated from leukocyte population based on their light scatter characteristics (*Figure 1*). Respiratory burst capacity was determined in the gated monocyte population by measuring the intensity of green fluorescence based on the conversion of non-fluorescent substrate (DHR) into fluorescent product under the influence of an intracellular reactive oxygen intermediate, namely superoxide anion (*Figure 2*).

Statistical analysis

The results were shown as DHR median fluorescence in patients and respective controls. Normal distribution of the data was tested using Kolmogorov-Smirnov test. The data analysis was performed using Mann-Whitney's test. The significance level was defined at $p < 0.05$.

Results

The study involved eleven type 1 patients, four children and seven adults. All children were male, while the adult patients consisted of four women and three men. Age ranged from 11 to 72 years (mean 39.2 years). Five patients were on ERT, while six patients were treatment naïve at the time of the study. The patients' characteristics (age at diagnosis, sex, chitotriosidase level, severity score index (SSI) (3–5), prior to ERT institution, type of mutation and ERT) are presented in *Table 1*.

Eleven control subjects were age and sex matched to the patients. There were three children

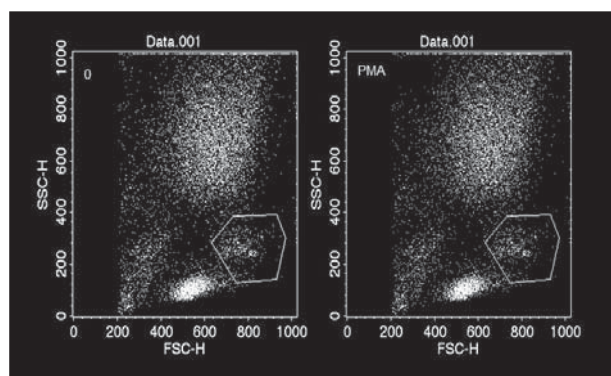


Figure 1 Dot plots represent forward and side light scatter of leukocytes isolated on Percoll gradient; polygonal regions encircle monocyte population before (0) and after (PMA) stimulation.

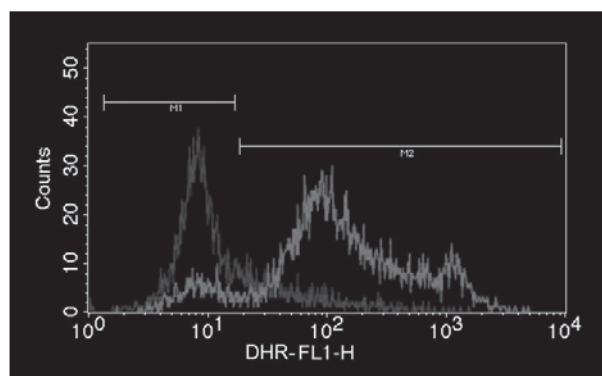


Figure 2 Histogram plots and overlay derived from monocyte regions of unstimulated (blue) and PMA stimulated (red) leukocytes.

Table I Patients' characteristics.

Patient ID	Age at diagnosis (years)	Sex	Chitotriosidase (nmol/mL/h) before ERT/now	SSI	Mutation	ERT
1	52	F	6700	7	N370S/?	No
2	61	M	5800	6	N370S/RecNci	No
3	48	M	11400	7	N370S/R463H	No
4	61	F	7500	8	N370S/N370S	No
5	41	F	1000	7	N370S/?	No
6	6	F	11500	8	N370S/ 55-BP	No
7	6	M	43500/1000	11	N370S/?	Yes
8	8	M	42700/2300	7	N370S/N370S	Yes
9	22	M	6200/2400	11	N370S/RecNci	Yes
10	6	M	2500/1000	12	N370S/R463H	Yes
11	4	M	24500/700	13	N370S/R463H	Yes

Legend: F = female, M = male.

(all male) and eight adults with even sex distribution. Age ranged from 11 to 60 years (mean 37.6 years).

The results are shown in *Table II*. There was no statistical difference in the median fluorescence among the patients and respective controls ($p > 0.05$) after PMA stimulation. Also, statistical difference was not reached among patients treated with ERT at the time and those untreated ($p > 0.05$).

Discussion

Infections in Gaucher patients are not universal, nor are there studies stating the exact prevalence of infectious comorbidities or complications in these patients. However, a few case reports and case series

motivated the researchers to additional studies concerning the functions of the cellular elements of innate and acquired immunity.

Literature search reveals a series of patients with osteomyelitis (6) which presents a differential diagnostic challenge especially related to bone crises, characteristic for Gaucher disease. Finkelstein et al. also described ten patients with osteomyelitis noting that it is a rare complication of the disease often caused by unusual and anaerobic bacteria (7).

Other rare infections mentioned in the literature are subdural empyema (8), pterygoid muscle candidiasis (9) and multiple foci of osteomyelitis and soft tissue abscesses in a patient with *Salmonella* sepsis (10).

Table II Flow cytometric analysis of oxidative burst in peripheral blood monocytes – results.

Subject	Age (years)	Sex	ERT	DHR median fluorescence (after PMA)
P1	54	F	No	99.1
C1	46	F		69.78
P2	61	M	No	122.98
C2	58	M		191.1
P3	50	M	No	92.64
C3	46	M		108.43
P4	72	F	No	46.14
C4	60	F		50.48
P5	51	F	No	26.42
C5	60	F		30.23
P6	31	F	No	40.68
C6	31	F		27.38
P7	13	M	Yes	43.71
C7	13	M		47.83
P8	11	M	Yes	48.26
C8	11	M		42.55
P9	56	M	Yes	42.55
C9	54	M		29.96
P10	17	M	Yes	482.61
C10	20	M		478.29
P11	15	M	Yes	239.28
C11	15	M		504.81

Legend: The patients are shown with their respective controls (P = patient, C = control); P1 matches C1, P2 matches C2 and so on, respectively (F = female, M = male).

Professional phagocytes (granulocytes, monocytes and macrophages) play a key role in host defense against infection, having a powerful molecular machinery which generates toxic oxygen radicals when activated. Among the best stimulators of phagocytosis are certainly formyl methionyl peptides released from bacteria at nanomolar concentrations at the site of infection, binding to specific receptors at the phagocyte membrane (11). Binding leads to chemotactic response manifested as endothelial cell adhesion, transendothelial transport and migration along a formyl peptide concentration gradient. The ultimate outcome of these events is engulfing bacteria i.e. phagocytosis by an endosomal vacuole. Phagocytosis brings into play another two important processes: respiratory burst, sudden activation of oxidative metabolism leading to superoxide anion generation, and degranulation – releasing the contents of cytoplasmic granules into the endosomal vacuole. The initial product of the respiratory burst is the superoxide anion (O_2^-) generated during mono-electronic reduction of oxygen:



The reaction is catalyzed by NADPH oxidase and requires constant repletion of NADPH via hexose monophosphate shunt. Other oxygen products generated during the respiratory burst are hydrogen peroxide and hydroxyl radical which generate hypochlorous acid and chloramines with significant bactericidal activity (11).

Monocyte dysfunction in Gaucher patients was previously studied by Liel et al. (12). The researchers found significant reduction in superoxide generation after stimulation by PMA, opsonized zymosan and formyl-methionyl-leucyl-phenylalanine compared to control subjects. Nitroblue tetrazolium reduction was also significantly reduced, as were antistaphylococcal bactericidal activity and phagocytosis. However, all those functions were spared in the granulocytes of the patients.

In a similar study by Marodi et al. (13), reduced bactericidal activity against viable bacteria was reported as well as reduced superoxide generation in mono-

cytes after stimulation with opsonized bacteria. Moreover, these processes were improved after treating the patients' monocytes with glucocerebrosidase.

In our study, there was no statistical difference among the monocytes of patients and control subjects. The statistical difference was also not reached between the patients receiving ERT and treatment naïve patients. Also, none of the patients manifested any serious infections apart from the usual illnesses such as common cold or urinary tract infection. However, our study was done using a different method, compared to the studies of Liel et al. (12) and Marodi et al. (13). In the former, the production of superoxide anion was measured as the superoxide dismutase inhibitable reduction of acetyl ferricytochrome c by the microtiter plate technique. The latter study used a similar method.

We used the method of flow cytometry. The general advantage of this method is that it enables simultaneous measurement of structural, biochemical and functional properties of single cells in suspension, at high speed (14). Using this method for measuring the respiratory burst is based on the conversion of non-fluorescent substrates into fluorescent products under the influence of intracellular oxygen species i.e. superoxide anion. A few different substrates can be used including DHR (15) which is a more sensitive indicator of respiratory burst compared to other indicator systems such as dihydroethidium (DHE)/ethidium bromide (EB) (16). Lymphocytes and other cell populations can be separated and quantified by the combined measuring of forward light scatter depending on cell size and the side light scatter which depends on cell granularity. Acquired graphic cytograms can be used afterwards for gating the desired cell populations (14).

The range of DHR median fluorescence among the controls and Gaucher Type 1 patients varies substantially. There is limited data describing the monocyte capacity to produce reactive oxygen species upon stimulation with PMA. This function may be affected by age and sex, as demonstrated previously although in a different read-out system (17). However, the study of Alvarez and Santa Marina (17)

included subjects aged 25–55 and lacks data for individuals before puberty and teenagers. Due to the small sample size, we cannot speculate whether the considerable differences in measured fluorescence observed between young subjects in our study are a reflection of the hormonal or any other developmental variability in those individuals.

Our method might be more accurate due to its convenience of the electronic gating of monocytes. The monocytes are separated from other leukocyte populations according to light scatter characteristics. Other methods of cell separation are based on surface marker expression or adhesion characteristics. These methods of separation might lead to cell activation introducing additional sources of variability into the test. On the other hand, if the respiration burst capacity is a reliable indicator of macrophage function, which in turn reflects the patient's clinical status regarding susceptibility to infection, the relatively small sample size of eleven patients who were mildly affected may not demonstrate such a correlation. Additionally, perturbation of normal physiological functions of lipid-laden macrophages may have triggered off a series of secondary events. Also, if the patients are on ERT or having some residual glucocerebrosidase activity due to mild mutations, the impact on macrophage respiration burst capacity may be lessened.

In conclusion, flow cytometry might represent a more accurate and more reliable measure of respiratory burst compared to the methods of other researchers. Respiratory burst disturbance in monocytes does not seem to contribute to the increased susceptibility to infection in Gaucher patients.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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