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A New Chemiluminescent Method for Evaluation of the Functional Activity of Neutrophils in Patients with Type 2 Diabetes Mellitus

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Functional activity of neutrophils was evaluated by the chemiluminescent method with successive double stimulation by soluble stimuli with different mechanisms of action: phorbol-12-myristate-13-acetate (PMA) and phormyl-methionyl-leucyl-phenilalanine (fMLP). The study was carried out in 26 patients receiving oral sugar-reducing therapy. In addition to the functional activity of neutrophils, the levels of TBA reactive products, inflammation markers, blood clotting values, and biochemical parameters were measured. The results showed mainly reduction of the granulocytic component of the immune system in the patients.

Key Words: *oxidative stress; neutrophil activity; kinetic chemiluminescence; type 2 diabetes mellitus*

The data on functional activity of neutrophils in diabetes mellitus (DM) are contradictory. It is assumed that the activity of NADPH oxidase is elevated in endothelial cells [6] and immune cells (monocytes) [8], while the neutrophils are in a priming state [11] and produce high amounts of H_2O_2 [5]. However, according to some data, the activity of neutrophils is reduced in patients with non-insulin-dependent DM2 [10] and in children with DM1 [1].

Various methods are used for evaluation of the neutrophil functional activity [2,7,9], for example, NBT test variants [4]. They are rather simple, but do not demonstrate the time course of free radical production by the cells and involve isolation of the phagocyte population.

The chemiluminescent (CL) method is simple, available, informative, and sensitive. It is widely used for evaluation of the radical-producing activity of cells. A novel approach to evaluation of the functional activity of whole blood neutrophils is developed at Department of Medical Biophysics of M. V. Lomonosov Moscow State University. It is based on registration of the time course of neutrophil fluorescence stimulated in succession by two substances with different mechanisms of action [3]. At stage 1 the neutrophils are stimulated by phorbol-12-myristate-13-acetate (PMA) with intracellular mechanism of action, and at stage 2 the primary stimulation is carried out: with formyl-methionyl-leucyl-phenilalanine (fMLP) with extracellular mechanism of action. Double stimulation evokes maximal complete response of neutrophils and hence, improves method sensitivity and makes its results more accurate. Analysis of the entire kinetic curve improves significantly the informative value of the method.

We study the functional activity of neutrophils by double stimulation CL method in patients with DM2.

MATERIALS AND METHODS

The study was carried out in 26 patients with DM2 (disease history 5.4±3.6 years) receiving oral sugar-reducing therapy. The mean age of the patients was 60.8±7.0 years. Complex clinical laboratory studies

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were carried out in all patients: evaluation of lipid metabolism, glycemic control, blood clotting, chronic inflammation and oxidative stress parameters (TBA reactive substances). The patients were selected for the study by the following criteria: age above 18 years, DM2, fasting glycemia of 6-10 mmol/liter, glycated hemoglobin <10%, with ALT, AST, and alkaline phosphatase levels surpassing the upper threshold normal values no more than 2-fold. Patients with hepatic and/or renal insufficiency, severe cardiac failure, receiving estrogens or oral contraceptives, with alcohol or narcotic dependence, with malignant tumors detected less than 5 years before the study, lung rale, pregnancy and lactation were rejected. All patients signed informed consent to participation in the study.

The method was previously used in examinations of 87 healthy volunteers aged 32.4±11 years and they constituted the control group.

Chemiluminescent analysis was carried out on a 12-cuvette chemilumonometer Lum12 (DISoft). The following reagents were used: luminol, KH_2PO_4 (Sigma), Hanks liquid with glucose without stain (M. P. Chumakov Institute of Poliomyelitis and Viral Encephalitides), stabilized 2 mM HEPES; fMLP, PMA (Sigma).

Whole blood was collected in vacutainers with heparin (final concentration 4.5%; 45 µl/ml solution in cuvette), put into cuvette with Hanks' saline and luminol in the final concentration of 45 µM, and spontaneous CL was recorded over 12 min, after which PMA was added (final concentration in cuvette 50 ng/ml). After 20-min incubation, fMLP (final concentration 10 µM) stimulation was carried out and induced CL response was recorded over at least 60 min.

Mean neutrophil activity and extinction coefficient were studied in patients with DM2 and compared with the corresponding values in the control group.

The data were statistically processed by Statistica software (StatSoft).

RESULTS

Chemiluminescence curves are plotted for 24 patients. Their shape is similar to curves plotted for normal subjects (Fig. 1). Curves with a "slow" flash are plotted for two patients (Fig. 1, *a*).

The neutrophil CL curve includes three parts: spontaneous CL, CL after PMA prestimulation, and CL after primary stimulation with fMLP. The following parameters can be calculated from the curve: amplitude of spontaneous CL (A_{sp}; CL intensity at min 10 of fluorescence), amplitude of PMA-stimulated CL (A_{PMA}; CL intensity 20 min after PMA addition), amplitude of fMLP-induced CL flash (A_1; intensity at the peak maximum), and CL intensity during rapid flash decline (A_2; measured 23 min after rapid flash peak). In some cases the chemiluminogram shows an additional "slow" flash (Fig. 1, *a*), with the amplitude included in A_2.

Three values are particularly interesting as clinical parameters: "slow" flash, the A_1 value standardized by neutrophil count n(A*=A_1/n), and fluorescence decline coefficient $K_d=A_2/A_1$.

Our data indicate that the "slow" flash is an evidence of significant activation of intense strain of the granulocytic component of immunity [3]. Its presence in two patients can be attributed to acute infections (erysipelas, acute respiratory disease) shortly before the examination.

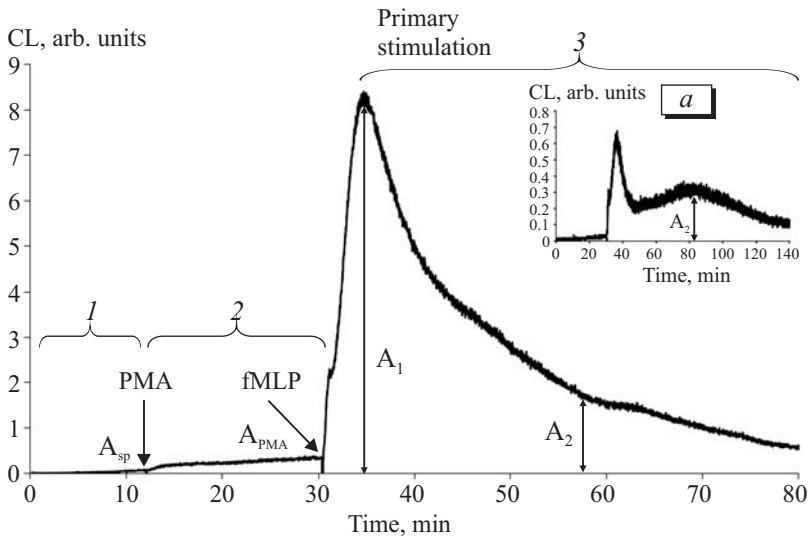

Fig. 1. Typical CL curve after double stimulation of neutrophils in the primaryity of patients with DM2 and normal subjects. *1*) Spontaneous CL; *2*) PMA prestimulation; *3*) primary fMLP stimulation. Arrows show time of addition of stimuli. *a*) "Slow" flash CL curve, plotted for a blood sample from DM patient.

The rapid flash amplitude, standardized by neutrophil count (A^*), can serve as a characteristic of the neutrophil specific activity. The data for control group (group A) were processed by the Shapiro–Wilk test for normality of distribution. The distribution differed significantly from the normal: n=87, W=0.93709, p=0.00037. The median was 4.06×10^{-5} B/cell, interquartile range (A) from 2.47×10^{-5} to 6.05×10^{-5} B/cell.

No appreciable differences between patients of different age or gender were detected in group A. We presumed that the same could be extrapolated to patients with DM (group B), and hence, analyzed the total sample (N=26). The median was 2.18×10^{-5} B/cell; interquartile range from 1.56×10^{-6} to 2.95×10^{-5} B/cell.

Groups A and B were independent, and hence, in order to verify the significance of differences between them, the data were compared by Mann–Whitney nonparametric test: U=397.0; Z=-3.533; p=0.0004. These data indicated that the difference between the neutrophil activities in the two groups was significant.

The decline coefficient was calculated as the proportion of fluorescence intensity at the maximum to the intensity 23 min after the maximum was attained (exactly the time when an extra slow flash could emerge). The parameters for K_d in group A were as follows: median 0.43, interquartile range 0.35-0.64; in group B: median 0.16, interquartile range 0.12-0.28. According to Mann–Whitney test, the differences between the groups were significant: U=182.0; Z=5.72; p<0.0001. Presumably, lower K_d values in the patients indicated more rapid exhaustion of neutrophils after exposure to the stimulus.

Comparison of the results in patients with DM and healthy volunteers allows the following conclusions. The functional activity of neutrophils in patients with DM2 is significantly lower than in normal subjects, this indicating insufficiency of the neutrophilic component of immune defense in the patients in many cases. It is known that for heretofore unknown reasons, DM patients are more susceptible to infections, particularly staphylococcal involvement of the skin and infections of the urinary tract. In addition, they are at a higher risk of socially significant infections (*e.g.*, tuberculosis). Presumably, our data will promote better understanding of the problem.

Hence, evaluation of the neutrophil functional activity in response to double stimulation can be used for studies of the immune system function in DM patients.

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