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COMPARATIVE STUDY OF THE CHEMILUMINESCENCE PRODUCED BY THE ACTIVATED POLYMORPHONUCLEAR LEUKOCYTES IN PHYSIOLOGICAL AND VARIOUS PATHOLOGICAL CONDITIONS

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ABSTRACT

The chemiluminescent emission released by activated polymorphonuclear leukocytes has been known for a long time, but its clinical use is still scarce. We suggest the as a quantitative measurement of chemiluminescence the stimulatory index (ratio of the chemiluminescence value of stimulated polymorphonuclear leukocytes, to non-stimulated leukocytes) obtained for the same individual. The results obtained by a standardised simplified technique for more than 2000 individuals (healthy persons such as medical personnel, athletes, soldier and patients suffering from cardiovascular disease, cancer and pneumoconiosis) explain the still restricted use of chemiluminescence. There was a wide range of individual variations and an overlap of normal physiological and pathological values. A significant statistical difference was obtained only for patients in the acute phase of the disease. The chemiluminescence measurement of phagocytic activity of leukocytes is strongly influenced by age, diet, presence of stress and chronic inflammations.

RESUMO

A emissão de quimiluminescência por leucócitos polimorfonucleares ativados é conhecida já faz muito tempo, porém as suas apilcações clinicas estão muito escassas. Este trabalho propõe como medida de quimiluminescência o índice estimulatório (razão do valor da quimiluminescência de leucócitos polimorfonucleares estimulados e não-estimulados) para um mesmo indivíduo. Resultados experimentais obtidos para mais de 2000 pessoas (alguns gozando de boa saúde como atletas. soldados, componentes do corpo médico e pacientes sofrendo de doenças cardiovasculares, câncer e pneumoconiose explicam o uso ainda restrito da quimiluminescência. Para um número grande de indivíduos aconteceu uma superposição de valores normais e patológicos. Uma diferença estatisticamente significativa foi observada somente no caso de pacientes na fase aguda da doença. A medida de atividade fagocítica de leucócitos usando quimiluminescência é altamente influenciada pela idade, dieta, presença de estresse e inflamações crônicas.

KEY WORDS: chemiluminescence, phagocytosis, leukocytes.

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INTRODUCTION

The phagocytic activation of oxidative metabolic pathways of polymorphonuclear leukocytes (PMNL) has been shown to yield electronically excited molecules and free radicals. These reactive oxygen species (ROS), either by themselves or by interaction with cellularcomponents, produce a long lasting emission of chemiluminescence (CL) [2, 3, 4]. In spite of the fact that Allen discovered the CL produced by activated PMNL in 1972 [1], its clinical utilisation is sill scarce [7, 16]. The lack of an internationally accepted standardisation of the procedure, the final expression of the result of the determination, use of various instrumental techniques, interference with ingested antioxidants or drugs and the wide range of individual values might explain the limited utilisation of CL for clinical purposes.

Depending on the particular requirements [8, 21] of an assay system whole [20], purified PMNL [1, 2, 3, 4, 7], alveolar macrophages [17], or natural killer cells [10] were used as a source of CL. The in vitro activation of the phagocytic cells was triggered by various stimuli such as: opsonized zymosan [1, 2, 3, 4, 7], or bacteria [18], phorbol myrimethionyl-leucyl-phenylalanine [6].

In previous studies [14, 16], we showed that the CL emission of human activated PMNLs in healthy individuals depends also on age and the presence of physical or psychical stress. We nowl propose a simplified procedure for the measurement of the CL emission of PMNL in order to make its use more convenient for large clinical scale utilisation.

MATERIAL AND METHODS

The study was performed on 200 healthy individuals, aged 20-70 years, of both sexes and varied occupations. These subjects had normal haematological patterns, including the total leukocyte count.

The study also included 1800 patients, mostly (1100) cardiac catheterised patients from Carle Hospital, Urbana USA, pneumoconiosis (300) and lung cancer (400) from clinics of occupational diseases and of Radiobiology, Fundeni Clinical Hospital, Bucharest, Romania.

The suspension of purified PMNL was obtained by using the two step Histopaque kit (Sigma Chemical Co. St. Louis, USA, No.1077). The final dilution of PMNL to 1 x 10^7 cells/ml was performed by using Hanks' balanced salt medium (Gibco Life Technol. grand Bland, New York).

We also used a simplified procedure: ten ml whole blood containing 10 units of heparin was centrifuged at 700 G for 10 minutes. The supernatant containing the plasma was discarded, and its volume was replaced with a cold solution of ammonium chloride 0.87 %. After thorough mixing, the whole hemolysate was centrifuged for 7 minutes at 700 G. This procedure was repeated and centrifuged for 5 minutes at the same speed. The colourless residue that contained only leukocytes was washed with Hanks' solution. Finally, a suspension of 1 x 10^7 cells/ml was obtained by using Hanks' solution for dilution.

For both procedures, the final suspension contained neutrophils, 90% for the first and 70% for the second. For both procedures the blue tripan exclusion test showed approx. 98% viable cells.

The determination of phagocytic capacity of purified PMNL consists in the following procedure: For each individual, two samples are used. In each of the tubes 100 µl leukocyte suspension (1 x 10^6 cells/ml) and 100 µl luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) 0.1 mmol were introduced. In the first tube, nonstimulated, 800 µl of Hanhs' solution was added while in the second, stimulated only 700 µl of Hanks' was added. Both samples are kept for 10 minutes at 37°C. Then, 100 µl opsonized zymosan 1 mg/ml was added only in the stimulated sample. Both samples were introduced in the counter or luminometer and the emission of CL was recorded for 30-60 minutes. The identical technique was used for both procedures. In healthy persons, the lymphocytes which account for approx. 20 % of total leukocytes do not produce CL under such conditions, thus are not interfering with the determination.

Zymosan was prepared by boiling fresh yeast in alkaline medium in the conditions described by the LKB-Wallac (application procedure Nos 513) and was opsonized afterwards with AB serum. The final suspension was adjusted to 10 mg/ml and was divided into portions of 1 ml and kept at -20 °C until utilisation.

A Beckman beta Scintillation Counter LS-3801 with special single photon monitor equipment was used for measuring the CL emission. The final result for both procedures was expressed as the stimulatory index (SI) of phagocytosis obtained from the ratio of the CL value at any time of the stimulated sample and the CL value from the same period of time of the nonstimulated sample. Thus for each individual, both procedures included two samples, both non-stimulated and stimulated and the ratio of the measurement for the same period of time provided the SI.

All data were transformed to logarithmic scale in order to fit a Gaussian pattern. The calculations were performed using a statistical package from BMDP Statistical Software Inc. The significance of the differences between each value presented by two groups was evaluated by the Student's t test with $\rho < 0.05$, considered statistically significant.

RESULTS

Comparison of the procedures for obtaining PMNL

The viability of PMNL is essential in order to obtain a reliable measurement of the CL emission. Therefore, the purification of PMNL should not be of long duration to harm the cells. This explains why the testing of viability of purified PMNL by trypan blue exclusion is so important.

As can be seen in Fig. 1, the difference between the known procedure and the simplified variant we proposed is clear-cut. The known procedure is based on a suspension of PMNL, where neutrophils are 90 % of the total count, while for the second procedure, these cells account only for 70 % for a healthy person. This explains why for the same number of cells (1 × 10^6 cells/ml), the first procedure yielded a higher CL. For

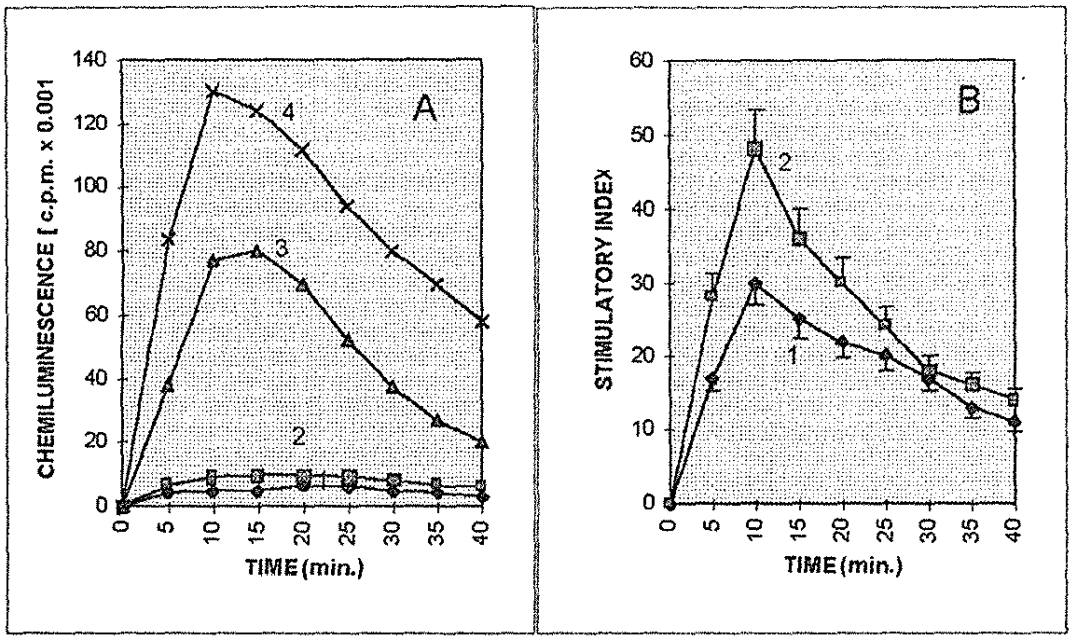

Fig. 1. - The kinetics of chemiluminescence produced by purified polymorphonuclear leukocytes.

A. The pattern of chemiluminescence mesurement for (2), non stimulated in the first procedure, and (1) in the second procedure, and stimulated in the first (4) and the second (3) procedure.

B. B The course development of the stimulatory index of phagocytosis for the second (1) and for the first (2) procedure.

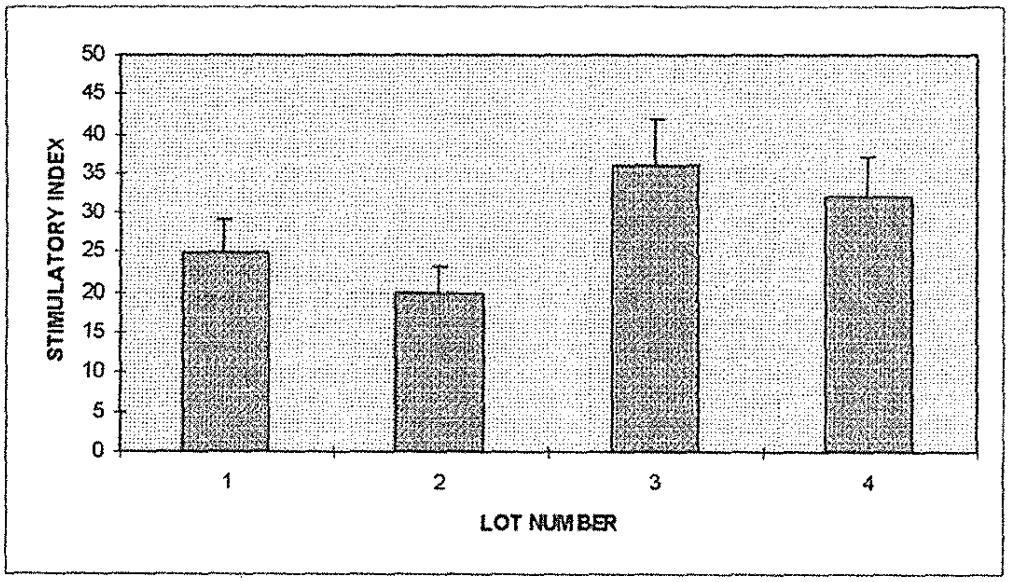

Fig.2 . - The variation range of the stimulatory index of polymorphonuclear leukocytes for healthy individuals based on measurement of the chemiluminescence emission according to the simplified procedure. The whole lot of healthy individuals consisted of four groups of 50 as follows: 1. Athlets (swimmers and bicycle runners), 2. Soldiers (18-20 years old), 3. Old persons (60-80 years), 4. Medical staff (medical doctors and nurses).

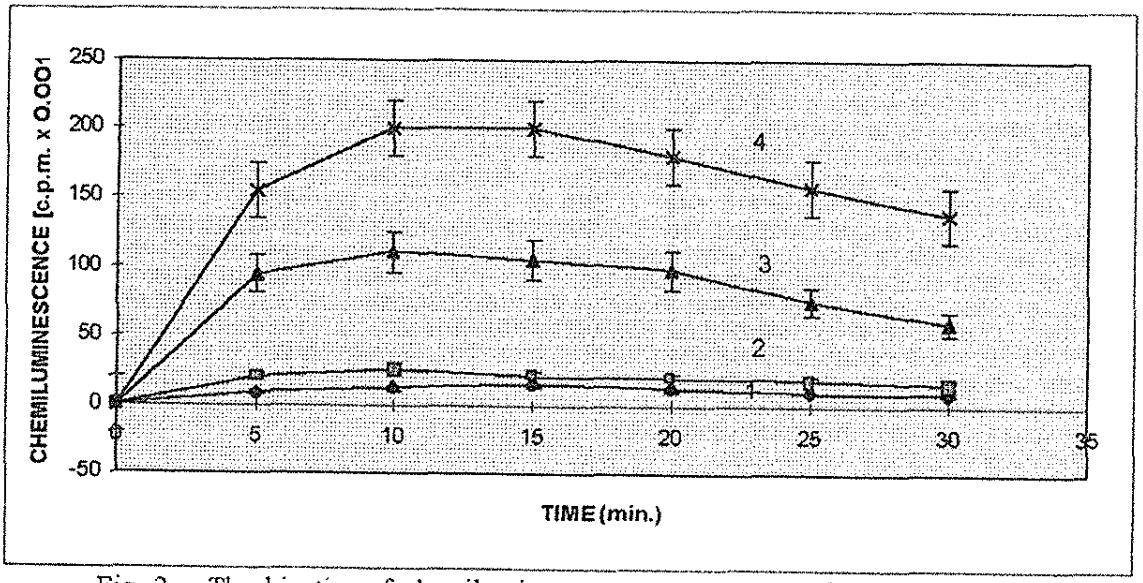

Fig. 3. - The kinetics of chemiluminescence measurement of phagocytosis in patients with pneumoconiosis.

Physiological range includes for normal individuals. (1) Non-stimulated and (3) stimulated values,

Patients with pneumoconiosis, (2) Non-stimulated and (4) stimulated samples.

The mean standard error is the result of measurements performed on 200 healthy individuals and 300 patients with pneumoconiosis.

TABLE 1 The variations of the stimulatory index (SI) of phagocytosis in some pathological conditions

CONDITION		NUMBER OF PATIENTS	STIMULATORY INDEX	SIGNIFICANCE $p <$
CONTROL		200	25 ± 7	
PNEUMOCONIOSIS		300	64 ± 14	0.01
LUNG CANCER		400	43 ± 18	0.08
CARDIAC CATHETERISED PATIENTS	**0 % stenosis**	340	53 ± 12	0.05
	80 - 100 % stenosis	760	61 ±17	0.01

nonstimulated samples, the two procedures provide no significant difference. As seen in Fig. 1B, where the CL measurement is expressed as the SI (stimularory index) the difference between procedures is smaller and statistically non significant. The first procedure is more time consuming and this makes it less attractive for a wide range of utilisation.

Physiological range of stimulatory index

The stimulatory index of phagocytosis is influenced by any chronic inflammation or stress condition. As presented in Fig. 2 in a whole lot of healthy individuals, the SI differs according to age, being significantly higher to older persons as compared with younger ones (athletes or soldiers).

The second observation is related to the occupational status of individuals. The medical staff, that include medical doctors and nurses, exhibits a higher statistically significant phagocytosis as compared with the athletes and soldiers groups. The variance of the individual values is also higher in the last group as compared with the others. The smallest variance was noticed for the soldiers group, which was the most homogeneous as compared with others, especially with the first one.

The determination of the stimulatory index in pathological conditions

Congenital deficiences of leukocytes, such as chronic granulomatosis disease (lack of respiratory burst during phagocytosis) or myeloperoxidase deficiency are the most widely known pathological conditions, where no CL emission is produced upon in vitro activation [7, 21]. But these diseases are rare, so CL utilisation is scarce.

Pneumoconiosis, known as inflammation of respiratory pathways and the lungs arises mostly following occupational exposure to mineral dust or asbestos.

As seen in Fig. 3, in patients with different forms of pneumoconiosis (asthma, silicosis), a significant increase of CL emission was observed. While the CL emission of nonstimulated samples might be overlapping with the physiological range, for the stimulated samples, the increased values are statistically different as compared with the healthy persons. For a significant number of patients investigated, the difference is clear cut only in the acute period of the disease.

The determination of CL emission was performed before treatment in order to avoid the strong influence of drugs.

As presented in Table 1, we extended our study on several groups of patients. In all pathological conditions, the SI was increased, but over a wide range. In pneumoconiosis, the data were found significantly increaseed as was the case for the patients with 100% stenosis.

For the patients with lung cancer, a wide range of modifications were noticed, but in most cases the individual variations were so high, that no statistical interpretation was possible. Similar wide ranges of modifications were noticed for patients with 0% stenosis, where the presence of acute inflammations might explain the chest pain, as well as the higher value of SI.

DISCUSSION

The obtention of data presented in our study performed on a large population of 2000 individuals was possible only by using a simplified procedure for purifying the PMNL. As shown in Fig. 1, our simplified procedure is simple, not expensive and reliable may be used for a large number of individuals.

As shown in Fig. 2, the data for healthy individuals are greatly influenced by many factors related to age, occupation and exposure to pathogens (medical staff) or stressful conditions (athletes, medical staff). The presence of chronic inflammatory ailments could not be ignored, especially for aged people or those exposed to pathogens (medical staff), mineral dust (miners, workers). Such observation may be extended to stressed people or with non defined pain as those with 0% stenosis.

For individuals with higher levels of phagocytosis, the increased levels of the SI suggest a non specific parameter with a strong individual character. As shown in Fig. 3 and Table 1 the analysis of the data for a lot of patients did not show its clinical value. The CL measurement of phagocytosis should be performed before treatment and as it possesses an individual trait. The variation of SI might offer the possibility to monitor the efficiency of the treatment and to evaluate the course of inflammations, which very often are present in a wide range of pathological conditions.

Such interpretation focused on individual level and the monitoring of the evolutive course of the disease and treatment is superior to the previous clinical interpretations [5, 7, 9, 11, 12, 13, 15, 19]. According to our data, the CL measurement of phagocytosis is related with the acute phase of diseases [22], as well as their chronic evolution. In almost all cases, smoking (present or within last 5 years) significantly increased the CL measurement. The SI is a dynamic parameter that mirrors the response of the phagocytic activity of leukocytes to various endogenous situations.

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