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Application of the chemiluminescence systems to evaluate the role of Fcγ and complement receptors in stimulating the oxidative burst in neutrophils

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Abstract

We have established a luminol- and a lucigenin-dependent CL methods to investigate the role of the receptors for Fc portion of IgG (FcγR) and/or complement receptors (CR) in mediating the oxidative burst in neutrophils from systemic lupus erythematosus (SLE) patients compared with healthy controls. In the luminol-CL system, all the reactive oxygen species (ROS) are responsible for light production, whereas in the lucigenin-CL system, only the first ROS generated, converts the lucigenin into an unstable intermediate molecule, which also emits light. First, neutrophils from healthy controls and SLE patients were stimulated with different IC opsonized or not with complement from normal human serum (NHS) or SLE serum, in presence of 10^{-4} M luminol. This method was able to differentiate the role of the FcγR, CR and FcγR/CR co-operation in mediating the oxidative burst, as well as show that the oxidative burst mediated by these receptors was reduced in neutrophils from SLE patients. Second, neutrophils from healthy controls and SLE patients were stimulated with different IC, opsonized or not with NHS, in presence of 10^{-3} M lucigenin. In this case, the lucigenin-CL system was also able to differentiate the role of FcγR and FcγR/CR co-operation, as well as show differences among healthy controls and two different groups of SLE patients according to their clinical manifestations. In conclusion, we have established two sensitive CL systems to study the role of FcγR and/or CR in stimulating the oxidative burst of neutrophils, which can be applied in monitoring the involvement of these receptors in the immunopathogenesis of SLE.

Keywords: Oxidative burst; Neutrophils; Chemiluminescent assays

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1. Introduction

Luminescence is a term used to describe the emission of light, which occurs when a molecule in an excited state relaxes to its ground state. In chemiluminescence (CL), the source of energy to obtain the excited state is a chemical reaction [1]. Then, CL can be defined in simplistic terms as chemical reaction that emit light [2].

Chemiluminescence has been one of the alternative labels as diagnostic and biochemical research tool [3]. In clinical chemistry, the CL systems have been widely used replacing the radioisotopes. Different classes of CL labels including luminol and lucigenin have been applied to biological assays. An exploited and important application of the luminol- and lucigenin-dependent CL is the study of reactive oxygen species (ROS) in relation with oxygen metabolism that is also known as oxidative burst of neutrophils.

Neutrophils are crucial in host defense against invading microorganisms through ROS production [4]. When neutrophils are activated, ROS released can transfer, via oxygenation, the chemiluminogenic probes (labels) luminol and lucigenin to an excited state and thus induce light emission from these labels. Various ROS (molecular oxygen, peroxides, superoxide anion) can oxidize luminol derivatives but catalysis either by enzymes or by mineral catalysts is required [5]. One of the biocatalysts that has been proposed is the myeloperoxidase, that is present in neutrophils. Luminol is exclusively oxygenated by hydrogen peroxide and/or species derived from it, while lucigenin may rather be oxygenated by the superoxide anion radical [6,7]. Moreover, with regard to lucigenin, this label has been used for specific quantification of cellular superoxide anion response after oxidative burst [8,9].

Detection and measurement of ROS production by neutrophils are of critical importance for investigating the pathophysiological consequences resulting from altered cellular mechanisms that lead to the oxidative burst. Immune complexes (IC) containing immunoglobulin G (IgG) bind to neutrophils and stimulate the NADPH oxidase complex, which leads to the oxidative burst characterized by production of ROS [4]. This binding is mediated by receptors for Fc portion of IgG (FcγR) and/or complement receptors (CR), when the IC are opsonized with fragments of complement proteins. The interaction IC-neutrophils, via FcγR and/or CR, might be impaired in autoimmune diseases, such as systemic lupus erythematosus (SLE), where these receptors, the complement system and high levels of circulating IC are involved in their immunopathogenesis [10]. FcγR and/or CR have been involved in mediating the tissue injury of the immune complex diseases such as SLE. These receptors can continuously stimulate the oxidative burst in neutrophils establishing a chronic inflammatory process. Then, we have established a luminol- and a lucigenin-dependent CL systems to study the role of the FcγR and CR in mediating the oxidative burst in neutrophils from SLE patients compared with healthy controls. In the present study, we present the standardization conditions and protocols to be applied.

2. Experimental

2.1. Patients and healthy controls

All patients fulfilled the American College of Rheumatology (ACR) classification criteria for SLE [11] and were reviewed for evidence of clinical and laboratory markers of lupus activity. Disease activity was measured using the SLE disease activity index [12]. Patients were diagnosed with SLE at the Hospital das Clínicas, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo (HCFMRP-USP). Age- and sex-matched healthy volunteers were studied as a control group. Local research ethics committee approval was obtained for the taking of blood samples and all studied patients agreed to provide them.

2.2. Immune complexes

Bovine serum albumin (BSA; Sigma A 7638) or Ovalbumin (OVA; Sigma A 5503) were used as antigen. Rabbit anti-BSA or anti-OVA IgG antibodies were used to prepare the IC formed at equivalence. It was also prepared IC from the

mixture of BSA and anti-BSA F(ab′)2 [the pepsin fragment of rabbit anti-BSA IgG]. These IC were used in the assay systems as suspensions.

2.3. Opsonization of immune complexes

Normal human serum (NHS) and SLE serum (SLES) were obtained from blood samples collected in the absence of anticoagulant and were used as complement sources. IC were incubated with NHS or SLES diluted 1:2 in veronal-buffered saline, containing 0.25 mM $CaCl_2$ and 0.83 mM $MgCl_2$, pH 7.2. After incubation at 37 °C for 30 min, cold PBS, pH 7.4, was added to the mixtures, which were then centrifuged at 4 °C for 10 min. The pellets containing the IC were washed once with phosphate buffered saline (PBS), pH 7.4, and resuspended in Hanks' balanced saline solution (HBSS), pH 7.2.

2.4. Neutrophils

Human peripheral blood was collected from healthy volunteers and from SLE patients using disodium ethylenediaminetetraacetate (EDTA-Na_2) as anticoagulant. Polymorphonuclear (PMN) neutrophils were isolated by a gelatin gradient [13]. Briefly, the blood sample was centrifuged at $755 \times g$ at 4 °C for 10 min. Polymorphonuclear neutrophils were separated from the pellet by addition of the 0.15 M NaCl solution containing 2.5% gelatin. This mixture was incubated at 37 °C for 30 min. The supernatant, containing neutrophils, was centrifuged at $755 \times g$ for 10 min and washed in PBS, pH 7.4, at $480 \times g$ at 4 °C for 10 min. The pellet was diluted in 0.83% NH_4Cl, pH 7.2, an erythrocyte lysing solution, and centrifuged at $480 \times g$ at 4 °C for 10 min. Neutrophils were washed in HBSS, pH 7.2, and diluted in HBSS/0.1% gelatin, pH 7.2, until use. This isolation procedure supplies 92% of neutrophils. All reagents and glassware were LPS-free when tested with E-TOXATE® (Sigma, product no. 210).

2.5. Chemiluminescence systems

Both chemiluminescence systems described in the present study were performed in a luminometer Bio-Orbit 1250 and the chemiluminescence production was recorded in millivolts (mV). The results were expressed as area under the curve of the chemiluminescence profile.

2.6. Luminol-dependent chemiluminescence

These assays were performed with BSA/anti-BSA immune complexes. The neutrophils diluted at 2×10^6/2 ml in HBSS/0.1% gelatin, pH 7.2, were mixed with different IC, in the presence of 10^{-4} M luminol. First, it was used different concentrations (10, 15, 30, 40 and 60 μg) of IgG-IC and IgG-IC/NHS, in order to establish the concentration able to stimulate the maximum of CL from 2×10^6 neutrophils. Second, it was used a concentration interval from 40 to 80 μg of IgG-IC/NHS and IgG-IC/SLES, to confirm if the maximum of CL could be observed when the stimulus (IC) were opsonized with SLE serum. When the conditions were established, it was performed the CL assays using 60 μg of the different IC [IgG-IC; IgG-IC/NHS, IgG-IC/SLES, F(ab′)2-IC/NHS or F(ab′)2-IC/SLES]. The reaction was kept at 37 °C for 15 min. Neutrophils incubated with luminol and HBSS in the absence of IC were used as control.

2.7. Lucigenin-dependent chemiluminescence

These assays were performed with OVA/anti-OVA immune complexes. The neutrophils diluted at 4×10^6/2 ml in HBSS/0.1% gelatin, pH 7.2, were mixed with 80 μg of IgG-IC or IgG-IC/NHS in the presence of 10^{-3} M lucigenin. The reaction was kept at 37 °C for 10 min. Neutrophils incubated with lucigenin and HBSS in the absence of IC were used as control.

2.8. Statistical analysis

Values are given as mean ± standard deviation (S.D.). Student's unpaired t-test was used to analyze the data. $P \leq 0.05$ was considered significant.

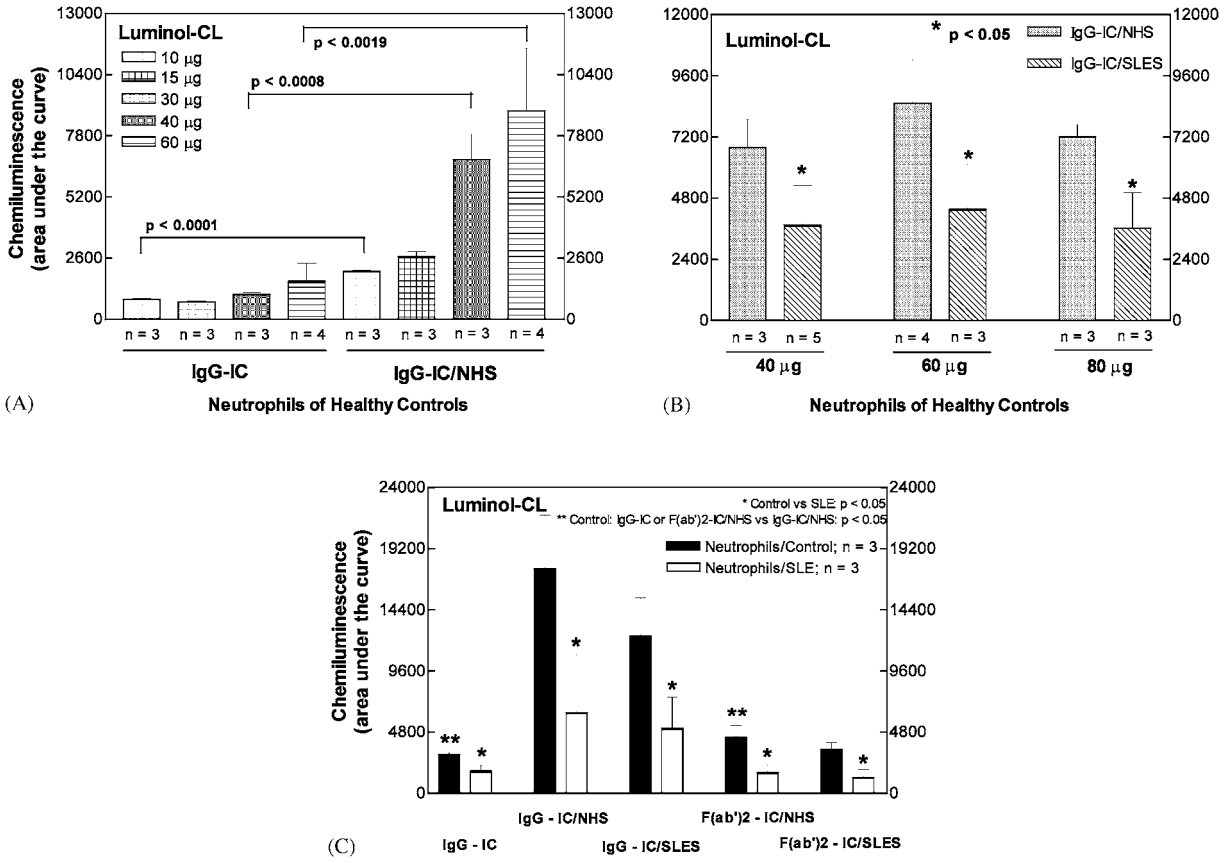

Fig. 1. Luminol-dependent CL system. Neutrophils (2×10^6) were stimulated with different concentrations of IC. The reaction was kept at 37 °C for 15 min in presence of 10^{-4} M luminol and recorded in mV. Cells and luminol in the absence of IC were used as background controls. These results are presented as the values of the mean ± S.D. of the areas under the curves of the CL profiles. (A) Curve dose–response of neutrophils of healthy controls stimulated with IgG-IC or IgG-IC opsonized with complement from NHS (IgG-IC/NHS). (B) Effect of the low level of complement in SLE patient sera on luminol-dependent CL. Neutrophils of healthy controls were stimulated with IgG-IC/NHS or IgG-IC opsonized with complement of SLE patient sera (IgG-IC/SLES). (C) Luminol-dependent CL of neutrophils, stimulated with 60 µg of different IC, of healthy controls and of neutrophils of active SLE patients, mediated by FcγR (IgG-IC), FcγR and complement receptors (IgG-IC/NHS, IgG-IC/SLES) and complement receptors [F(ab′)2-IC/NHS, F(ab′)2-IC/SLES]. Statistical differences: (B) IgG-IC/NHS vs. IgG-IC/SLES: $*P \leq 0.05$; (C) Neutrophils/SLE vs. Neutrophils/Control: $*P \leq 0.05$ and Neutrophils/Control with IC-IgG or Neutrophils/Control with F(ab′)2/NHS vs. Neutrophils/Control with IgG-IC/NHS: $**P \leq 0.05$ (Student's unpaired t-test).

3. Results and discussion

In the luminol-CL system, all the ROS convert the luminol into the aminophtalate anion, which is inherently unstable and its return to the ground state is accompanied by the release of photons. This light production, or CL, is one of the characteristic of the oxidative burst. With regard to lucigenin-CL system, only the superoxide anion, the first ROS generated, converts the lucigenin into an unstable intermediate molecule, which also emits light when returns to the ground state [3]. The interest in these different CL systems is in providing facilities to measure different ROS during the oxidative burst. The possibility to study neutrophil metabolism by CL has been widely

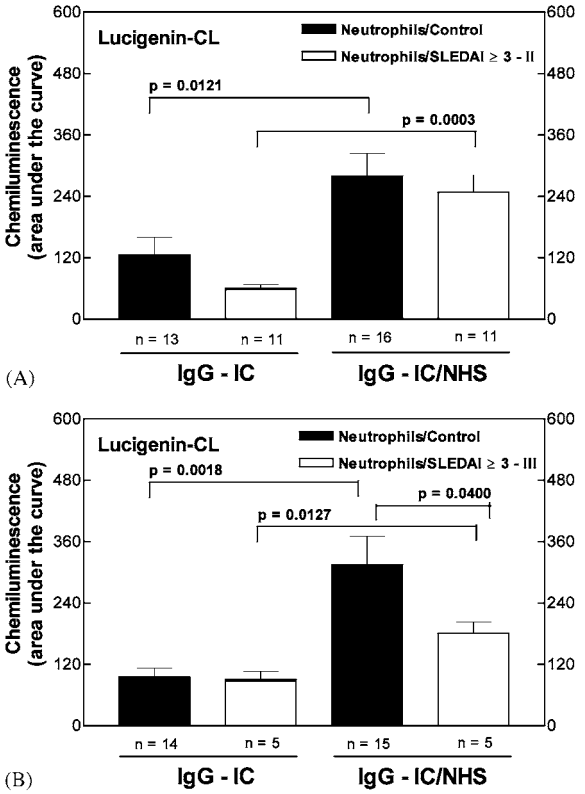

Fig. 2. Lucigenin-dependent chemiluminescence system. Neutrophils (4×10^6) of healthy controls and of active SLE patients were stimulated with 80 μg of immune complexes (IC) containing IgG (IgG-IC) or with IgG-IC opsonized with complement from NHS (IgG-IC/NHS). The reaction was kept at 37 °C for 10 min in presence of 10^{-3} M lucigenin and recorded in mV. Cells and lucigenin in the absence of IC were used as background controls. These results are presented as the values of the mean ± S.D. of the areas under the curves of the chemiluminescence profiles. Neutrophils of healthy controls were compared with neutrophils of active SLE patients with SLEDAI ≥ 3/Type II (A) and with neutrophils of active SLE patients with SLEDAI ≥ 3/Type III (B) Statistical differences: $^*P \leq 0.05$, Student's unpaired t-test.

exploited in the last years. The intensity of CL production has been a measurement to differentiate acute from chronic diseases [14–20]. It is possible that alterations of neutrophil metabolism be of clinical significance [21].

We have previously established protocols of luminol-dependent CL to study the oxidative burst of rabbit neutrophils stimulated with IC with different antibody functional affinities [13]. A modification of this method has been applied in an attempt to investigate the role of FcγR and the complement receptors in mediating the oxidative burst of neutrophils in physiological and pathological conditions.

The CL are expressed in area under the curve in order to represent the total response over a time period. Concerning luminol CL assay, this time is 15 min, because the measurement of CL production is more lasting than that measured by lucigenin. In lucigenin CL assay, after 10 min, it is not possible to measure a significant CL response.

In Fig. 1A, the results show that the luminol-dependent CL is a sensitive method to differentiate the oxidative burst of neutrophils mediated only by Fcγ receptors (IgG-IC) from that mediated by co-operation of Fcγ and complement receptors (IgG-IC/NHS). The curve dose-response shows that the CL production is dependent on the presence of membrane neutrophil receptors, i.e. the increase of the concentration of IC, increases the CL production until it reaches a maximum. Moreover, the luminol-dependent CL system is also able to differentiate the oxidative burst of neutrophils stimulated with IC opsonized with complement from NHS (IgG-IC/NHS) from that when the neutrophils are stimulated with IC opsonized with complement from SLE serum (IgG-IC/SLES), which presents low level of complement proteins (Fig. 1B).

The results presented in Fig. 1A, B gave us the conditions to establish a protocol to study the luminol-dependent CL response of neutrophils of SLE patients, which were with the disease in activity (active SLE), mediated by Fcγ and/or complement receptors. The Fig. 1C shows the response of neutrophils of SLE patients compared with neutrophils from healthy controls, when the cell responses were stimulated by different immune complexes. It is possible to evaluate the individual role of the Fcγ (IC-IgG) and the co-operation of FcγR and complement receptors (IgG-IC/NHS) [(Fig. 1A, statistical differences: IgG-IC vs. IgG-IC/NHS, $P \leq 0.05$, for 10, 40 and 60 μg of IC) and (Fig. 1C, statistical differences for neutrophil controls: IgG-IC vs. IgG-IC/NHS, $P \leq 0.05$)], as

Table 1
Maximum CL produced by neutrophils

Immune complexes (IC) (stimulus)	T_{max} (min)[a]		CL_{max} (mV)[b]	
	Control ± S.D. (*n*)	SLE ± S.D. (*n*)	Control ± S.D. (*n*)	SLE ± S.D. (*n*)
Luminol				
IgG-IC	3 ± 3 (3)	5 ± 2 (3)	332 ± 37 (3)*	222 ± 25 (3)
IgG-IC/NHS	5 ± 4 (3)	3 ± 2 (3)	1731 ± 875 (3)	764 ± 351(3)
IgG-IC/SLES	4 ± 3 (3)	2 ± 2 (3)	1406 ± 660 (3)	626 ± 92 (3)
F(ab′)2-IC/NHS	5 ± 3 (3)	5 ± 3 (3)	404 ± 87 (3)*	151 ± 48 (3)
F(ab′)2-IC/SLES	3 ± 3 (3)	7 ± 4 (3)	357 ± 88 (3)*	117 ± 56 (3)
	Control	SLE (SLEDAI ≥ 3-II)	Control	SLE (SLEDAI ≥ 3-II)
Lucigenin				
IgG-IC	0.8 ± 0.3 (13)	1 ± 1 (11)	29 ± 6 (13)**	19 ± 2 (11)**
IgG-IC/NHS	1 ± 3 (13)	1 ± 1 (11)	100 ± 70 (13)*	52 ± 24 (11)
	Control	SLE (SLEDAI ≥ 3-III)	Control	SLE (SLEDAI ≥ 3-III)
IgG-IC	3 ± 3 (12)	0.9 ± 0.3 (5)	32 ± 27 (12)**	22 ± 10 (5)
IgG-IC/NHS	1 ± 1 (13)	0.9 ± 0.1 (5)	61 ± 33 (13)**	44 ± 23 (5)

n, number of samples. Values are expressed as mean ± S.D. Student's unpaired *t*-test. * Control vs. SLE: $P \leq 0.05$. ** In the same population cells, IgG-IC vs. IgG-IC/NHS: $P \leq 0.05$.

[a] T_{max}, time interval from the incubation of neutrophils and IC (zero time) to the time of the maximum rate of CL emission.

[b] CL_{max}, maximum rates of chemiluminescence recorded value in millivolts (mV) in T_{max}.

well as the individual role of the complement receptors [F(ab′)2-IC/NHS (Fig. 1C), statistical differences for neutrophil controls: IgG-IC/NHS vs. F(ab′)2-IC/NHS, $P \leq 0.05$].

In Fig. 1B, it was possible to observe a significant decrease of the oxidative burst of neutrophils from healthy controls, when this response was mediated by IgG-IC/SLES (statistical differences: IgG-IC/NHS vs. IgG-IC/SLES, $P \leq 0.05$, in all concentrations of IC tested). This result can reflect the sensitivity of this method for the number of ligands for the cell receptors presented in the immune complexes, since the SLE serum (SLES) can be defective in coating of the IC with complement [10].

The present results can be important because this method is also able to evaluate the difference between the CL response of neutrophils of healthy controls and neutrophils of SLE patients. Fig. 1C shows a significant decrease of the oxidative burst of neutrophils of active SLE patients compared with controls ($P \leq 0.05$), when this response was mediated by FcγR (IgG-IC), FcγR and CR (IgG-IC/NHS or IgG-IC/SLES) or only by CR [F(ab′)2-IC/NHS or F(ab′)2-IC/SLES].

With regard to the lucigenin-dependent CL system, the data in Fig. 2A, B show that this system does not seem able to differentiate the response of neutrophils of healthy controls from the neutrophils of SLE patients when the oxidative burst is mediated only by FcγR (IgG-IC). No significant differences were found when neutrophils from SLE patients with SLEDAI ≥ 3/Type II (disease activity index), as shown in Fig. 2A, and SLE patients with SLEDAI ≥ 3/Type III, as shown in Fig. 2B, were compared with healthy controls.

However, we observed that the lucigenin system is able to differentiate the oxidative burst of neutrophils mediated by Fcγ receptors (IgG-IC) from that mediated by co-operation of FcγR and complement receptors (IgG-IC/NHS). These results are presented in Fig. 2A, B where the CL response of the same population of neutrophils (controls and SLE patients with SLEDAI ≥ 3/Type II or SLEDAI ≥ 3/Type III) stimulated by

IgG-IC was compared with that stimulated by IgG-IC/NHS (statistical differences: IgG-IC vs. IgG-IC/NHS, $P \leq 0.05$).

Interestingly, when we evaluated the lucigenin-dependent CL responses between neutrophils from healthy controls and SLE patients, a significant decrease of the oxidative burst was observed only in neutrophils from SLE patients with a high disease activity index (Fig. 2B, SLEDAI $\geq$ 3/Type III vs. control, $P \leq 0.05$), when mediated by Fcγ receptors and CR (IgG-IC/NHS). These patients presented clinical manifestations of cytotoxicity and immune complex disease, whereas the other group, with SLEDAI $\geq$ 3/Type II presenting only clinical manifestations of immune complex disease, was not different from controls. This method appears thus suitable to distinguish the CL responses, in a same population of cells, when mediated only by FcγR (IgG-IC) to that mediated by FcγR and complement receptors (IgG-IC/NHS) (Fig. 2A, B). Although it is not able to differentiate the responses mediated only by FcγR (IgG-IC), between neutrophils from healthy controls and from SLE patients (Fig. 2A, B), the lucigenin system is able to differentiate the CL responses mediated by Fcγ receptors and CR (IgG-IC/NHS) between neutrophils from controls and SLE patients, when there is a more severe manifestation of disease activity SLEDAI $\geq$ 3/Type III (Fig. 2B).

With respect to the CL, the area under the curve expresses the total CL production by cells in specific conditions (stimulus vs. time period), i.e. it represents the total response over a time period. It would be possible to express the CL results in mV if we presented all CL profiles of each control and patient. In this case, it is not appropriate because there are too many controls and patients. However, it is possible to evaluate the kinetics of CL generation calculating the T_{max} and CL_{max}. T_{max} reflects the rate of binding of stimulus to the cells, i.e. the time interval from the incubation of neutrophils and stimulus to the time of the maximum rate of CL emission (CL_{max}). CL_{max} expresses the maximum rate of CL recorded in mV at T_{max} and represents a value for a point of the curve. CL_{max} can also reflect the efficient binding of the stimulus to the cells necessary to induce the NADPH-oxidase activity. Table 1 summarizes data giving the T_{max} and CL_{max} values recorded in the luminol and lucigenin assays. The kinetic parameters in Table 1 do not represent the same parameters as those reported in Figs. 1 and 2. Consequently, they must be considered separately.

In this work we demonstrated that the luminol- and lucigenin-dependent CL systems are sensitive methods to study the oxidative burst of neutrophils of SLE patients mediated by Fcγ and complement receptors, as described above.

In conclusion, the study of neutrophil CL has led to a better understanding of inflammatory processes and oxygen metabolites related diseases [22–24]. The CL systems, described in the present study, can be important tools to evaluate the specific role of the FcγR and/or CR in mediating the oxidative burst in both physiological and pathological conditions. Moreover, these methods can be applied in monitoring the functions of these receptors in the immunopathogenesis of SLE.

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