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Effect of low-dose prednisone in vivo on the ability of complement receptor to mediate an oxidative burst in rat neutrophils

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Abstract

Glucocorticoids have been used in the treatment of a variety of inflammatory processes including autoimmune diseases. However, the influence of low-dose glucocorticoids on the respiratory burst activity of neutrophils has not been studied. The aim of this work was to study the effect of treatment with low-dose prednisone on the oxidative burst of rat peripheral blood neutrophils. Wistar male rats were treated with prednisone by gavage (28, 87 or 257 μg/animal/day) for 7 or 15 days. These doses are equivalent to 10, 30 or 90 mg/adult human (~ 70 kg)/day, respectively. Sera from normal rats were used to opsonize zymosan (opZy). Neutrophils (1×10^5) were stimulated by opZy and the oxidative burst of control or treated rat cells was measured by luminol-dependent chemiluminescence (CL). Prednisone did not affect the CL of rat neutrophils for either period of treatment, or any studied doses, when compared with controls. These results suggest that the low-dose prednisone has no effect on the oxidative burst mediated by complement receptors during the rat neutrophil phagocytosis of complement-opZy.

Keywords: Glucocorticoids; Respiratory burst; Neutrophils; Complement receptor

Abbreviations: AUC, area under the curve; C3b, complement component 3b; CL, chemiluminescence; CR1, complement receptor type 1; CR3, complement receptor type 3; EDTA-Na, sodium ethylene-diaminetetraacetate; Fab, fragment antigen binding; Fc, fragment crystalline; FcγR, Fc gamma receptor; IC, immune complexes; Log, logarithm; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; opZy, opsonized zymosan; PMN, polymorphonuclear; SLE, systemic lupus erythematosus

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1. Introduction

Neutrophils are important effector cells in host defense against microorganisms. However, these cells have been implicated in mediating much of the tissue damage associated with chronic inflammatory diseases.

Glucocorticoids are known to suppress the inflammation process and are often used in the treatment of autoimmune diseases. They have many inhibitory effects on neutrophil functions such as chemotaxis (Hirasawa et al., 1992; Salak et al., 1993; Llewellyn-Jones et al., 1994), adhesion (Schleiner et al., 1989; Yoshida et al., 1997), transmigration (Youssef et al., 1996), apoptosis (Liles et al., 1995; Cox and Austin, 1997; Nittoh et al., 1998), oxidative burst (Fukushima et al., 1990; Tsuji and Shioya, 1994) and phagocytosis (Petroni et al., 1988; Direnzo et al., 1994). One of the most important adverse effects of glucocorticoids is increased susceptibility to infections and is related to the dose, dose interval and biologic half-life of the drug administered (Dale and Petersdorf, 1973; Williams et al., 1974; Fauci et al., 1976; Dannenberg, 1979; Goldstein et al., 1979). The mechanisms by which glucocorticoids exert these effects on neutrophils are not fully understood.

In phagocytes, hydrocortisone has been shown to inhibit the complement component 3b (C3b) receptor function of monocytes (Schreiber et al., 1975) and to affect the binding capacity of human neutrophil Fc and C3b receptors (Forslid and Hed, 1982).

Forslid and Hed (1987) demonstrated that hydrocortisone in high doses not only affects the mobilization of the complement receptor 1 (CR1/CD35) and CR3 (CD11a/CD18; Mac-1) but also affects the phagocytic process of activated granulocytes primarily by inhibiting the ingestion process.

We have observed a decreased oxidative burst in neutrophils of patients with systemic lupus erythematosus (SLE) (Marzocchi-Machado et al., 1998). We compared the chemiluminescence (CL) of neutrophil phagocytosis of IgG immune complexes (IC), opsonized IC and opsonized IC [F(ab′)2]. The CL responses of SLE neutrophils were lower than those of normal cells when mediated by Fc gamma and/or complement receptors. These patients were taking low-dose prednisone at the time of assessment (5–30 mg/day). There are relatively few studies of the effects of low-dose glucocorticoids on the neutrophil complement receptor functions. The aim of this work was to study the oxidative burst mediated by complement receptors of neutrophils from rats subjected to low-dose prednisone.

2. Materials and methods

2.1. Animals

Wistar male adult rats weighing ±200 g, bred in our own house-breeding program, were used in all experiments. All animals received water and food ad libitum and they were maintained at a controlled temperature room (25°C). These animals supplied neutrophils and serum to functional tests.

The number of animals used was: 12 treated (and 10 control) with 28 μg (7 and 15 days), 85 μg (15 days), and 257 μg (7 days), and 6 treated (and 6 control) animals with 85 μg (7 days) and 257 μg (15 days). Fewer animals were used in some cases because of the limited number of animals in our house-breeding program. The large discrepancy in animal numbers among the results is due to the fact that rat blood was obtained by decapitation, and many times the blood clotted quickly when contacted with animal's coat, which was impossible to avoid. Moreover, sometimes the number of neutrophils obtained from whole blood was not enough and so the number of animals was reduced in some groups.

2.2. Prednisone treatment

Two groups of animals were treated with prednisone for 7 and 15 days. Within each group there were three different doses tested. The rats treated with prednisone received daily, by gavage, doses of 28, 85 or 257 μg/animal during the treatment period. The control animals received water during the same period of their respective experimental group. After the desired periods the animals were killed by decapitation and the neutrophils were isolated from whole blood.

2.3. Neutrophils

Rat whole blood was collected from control and treated animals in sodium ethylene-diaminetetraace-

Table 1
Maximum CL produced by rat neutrophils
Values are expressed as medians. Mann–Whitney *U*-test (control vs. treated: no significant differences).

Dose/days	T_{max} (min)[a]		CL_{max} (log)[b]	
	Control	Treated	Control	Treated
28 μg/7 days	9.0	9.0	1.89	1.93
28 μg/15 days	6.0	5.0	6.0	5.5
87 μg/7 days	7.0	9.0	1.44	1.63
87 μg/15 days	6.0	5.5	2.37	2.25
257 μg/7 days	6.5	7.5	1.79	1.70
257 μg/15 days	8.0	6.5	1.47	2.31

[a] T_{max}: time interval from the incubation of rat neutrophils and opZy (zero time) to the time of the maximum rate of CL emission.
[b] CL_{max}: logarithm of maximum rates of chemiluminescence recorded value in millivolts in T_{max}.

tate (EDTA-Na) anticoagulant. Polymorphonuclear (PMN) neutrophils were isolated using a NIM[2] gradient (Cardinal™ Associated). Two gradient materials (NIM 2A and NIM 2B) and an erythrocyte lysing buffer compose this system. The 2B gradient was carefully poured on 2A gradient in equal volumes.

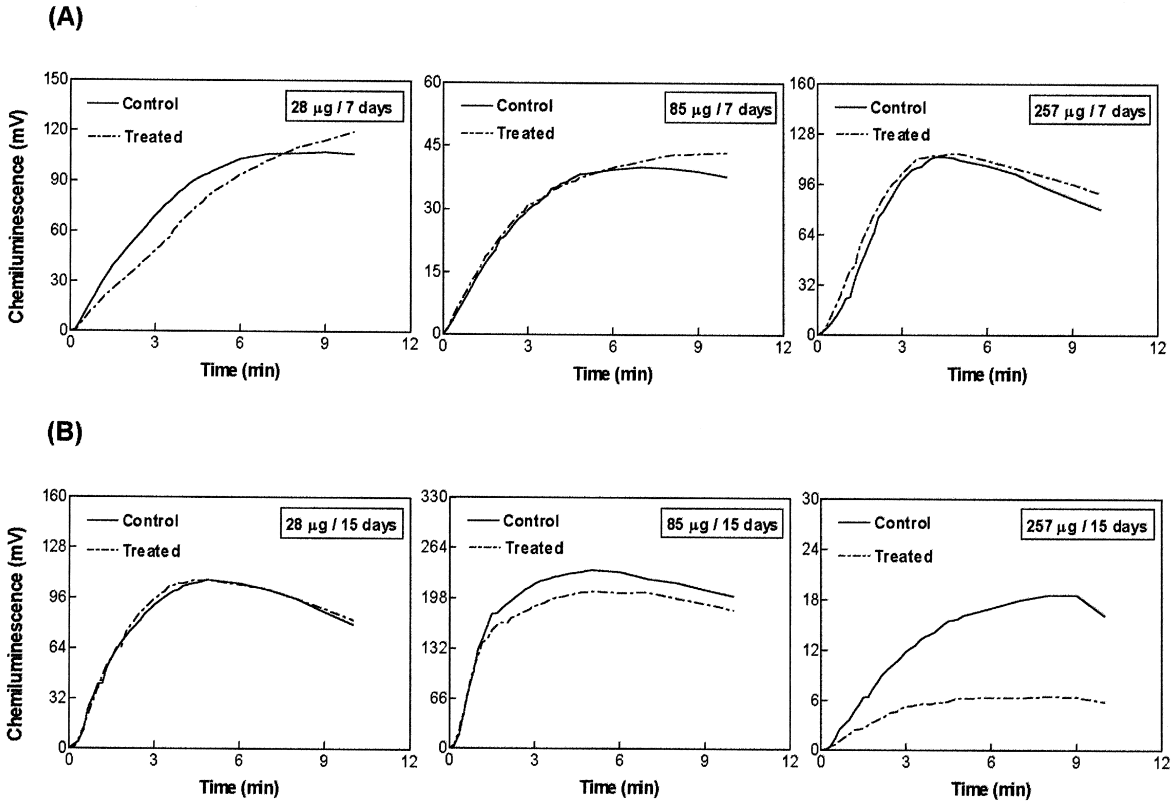

Fig. 1. Representation of kinetics of CL production by rat neutrophils stimulated with opZy. The animals were treated with different low-dose prednisone for 7 (A) or 15 (B) days. Neutrophils (1×10^5) were incubated with 2 mg opZy in 2 ml Hanks' solution pH 7.2, 0.1% gelatin and 10^{-4} M luminol. Data are presented as millivolts/minutes (mV/min).

The fresh anticoagulated rat blood was added to the upper gradient. The tube containing the gradients and blood was sealed and centrifuged at $900 \times g$ for 45 min in a swinging bucket rotor. The layer containing neutrophils was collected and washed in 0.15 M NaCl and centrifuged at $400 \times g$ for 10 min. The pellet was diluted in erythrocyte lysing buffer, centrifuged at $400 \times g$ for 10 min. Neutrophils were washed in Hank's balanced solution pH 7.2 and diluted in this buffer until use.

Viability tests carried out with trypan blue indicated that no more than 8% of nonviable cells were present in suspensions used in the experiments.

2.4. Opsonization of zymosan

Zymosan was opsonized according Cheung et al. (1983). To opsonize zymosan 400 μl of a serum pool from untreated rats was used. In the tests, serum pool was diluted 1:2 in veronal-buffered saline, containing 0.25 mM $CaCl_2$ and 0.83 mM $MgCl_2$, pH 7.2. A final concentration of 1 mg/ml of opZy was used in each test as stimulus to neutrophils.

2.5. Luminol-dependent CL

The CL generation is a sensitive indicator of the oxidative metabolism (Allen et al., 1972) and the assays were performed as described by Cheung et al. (1983). The neutrophils diluted at 1×10^5/ml in Hanks' balanced solution pH 7.2, were mixed with an equal volume of opZy suspension at 2 mg/ml (final concentration 1 mg/ml), in the presence of 10^{-4} M luminol. The reaction was monitored in a luminometer (Bio-Orbit 1250) at 37°C for 10 min and recorded in millivolts. The results are expressed

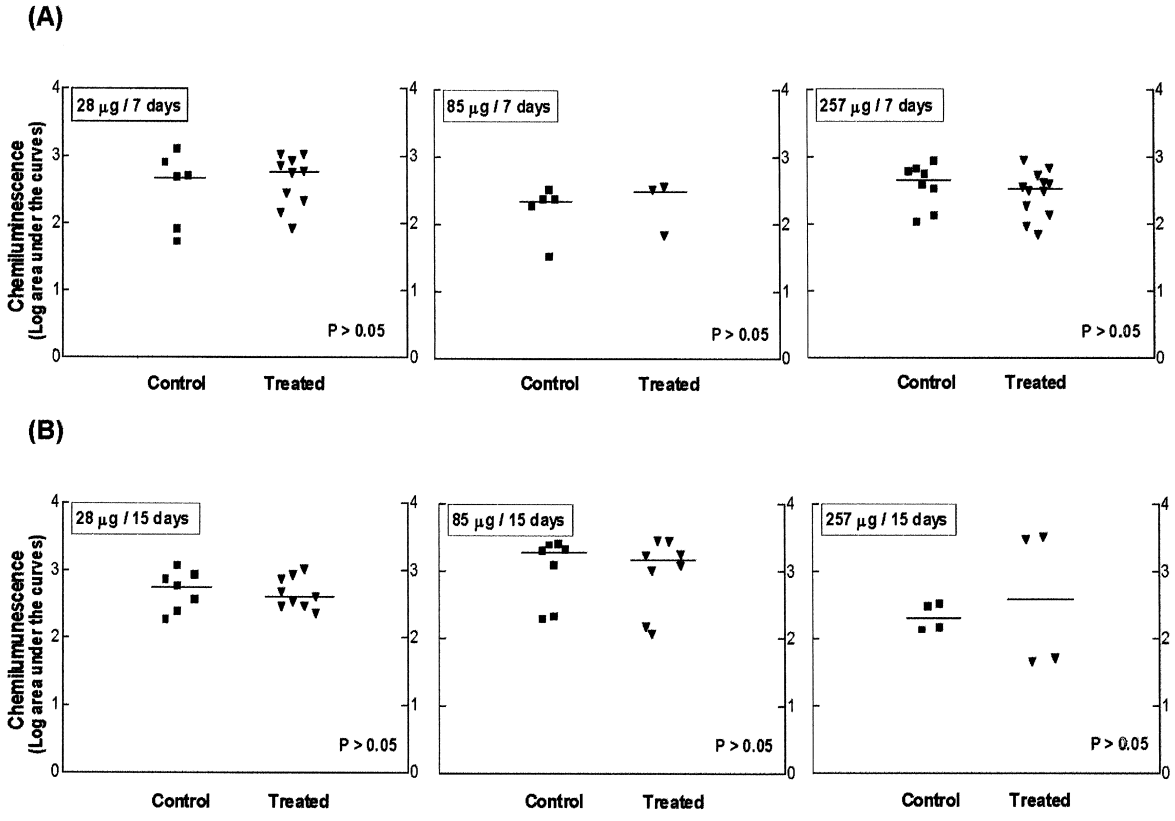

Fig. 2. Effect of different low-dose prednisone on cellular CL of rat neutrophils. Cells (1×10^5) were stimulated with 2 mg opZy in 2 ml Hanks' solution pH 7.2, 0.1% gelatin and 10^{-4} M luminol at 37°C for 10 min. CL was calculated as log of AUC of the CL profile. Periods of treatment: 7 (A) and 15 days (B). Data are medians from control and treated cells. No significant differences could be observed. Statistics: Mann–Whitney *U*-test.

as the logarithm (log) of maximum rates of CL recorded value in millivolts (Table 1) and as the log of area under the curves of the CL profile (Fig. 2). The CL profile is represented in Fig. 1 as millivolts/minutes.

2.6. Statistical analysis

Statistical analysis of the difference between control and treated animals was performed by the Mann–Whitney *U*-test for nonparametric data. $P < 0.05$ was considered significant.

3. Results

3.1. The maximum of CL produced by rat neutrophil treated with low-dose prednisone

Table 1 summarizes data giving some kinetic parameters of CL production by rat neutrophils using opZy as stimulating agent. No significant differences could be observed between control and treated samples for the slopes of the CL response and time-to-peak values (Mann–Whitney *U*-test).

3.2. Kinetic of CL production by rat neutrophil treated with low-dose prednisone

Kinetic of CL production from each experimental protocol is represented in Fig. 1 as millivolts/minutes (mV/min) and it is only from one animal.

This figure shows a representation of CL profiles between treated and control neutrophils at low and high dose. These results indicated the same efficiency of stimulus binding to the cells to induce the NADPH-oxidase activity. However, there could be differences in the complement receptor expression and/or function, even though the total CL produced were similar.

3.3. Effect of low-dose prednisone on cellular CL

CL values were calculated by logarithm (log) of the area under the curve (AUC) of the CL profile over a time period of 10 min. The absolute values of the AUC for the control and treated cells with their medians are presented in Fig. 2A and B.

Our data show that prednisone in all studied doses (28, 87 and 257 μg/animal/day) and both periods of treatment (7 and 15 days) had no significant effect on oxidative burst of rat neutrophil during phagocytosis of complement-opZy particles (Mann–Whitney *U*-test).

4. Discussion

The pivotal role of neutrophils in the host defense against bacterial infection is mediated by effector functions such as phagocytosis, degranulation of proteases and their ability to generate reactive oxygen derivatives. The phagocytosis can be triggered by receptors for the Fc region of IgG (FcγR) and the receptors of C3b and iC3b (CR1 and CR3). There are synergistic functions between FcγR and CR3 during the phagocytic response of human neutrophils (Okuro et al., 1995) as well as CR1 and CR3 (Sutterwala et al., 1996). The stable adhesion of complement-opsonized particles to cells expressing CR1 and CR3 is actually a dynamic molecular process in which an important function of leukocyte CR1 is to generate the ligands for CR3 (Sutterwala et al., 1996).

CR1 on PMN cells has been supposed to promote the adherence of the particles to the membrane of PMN, while Fcγ receptors enhance the endocytosis (Mantovani, 1975; Newman and Jonhston, 1979). Melamed et al. (1982) demonstrated that CR1 also has been found to promote superoxide generation and the release of lysosomal enzyme from PMN.

Although neutrophils are important in host defense, they are also major effectors of tissue damage through their ability to maintain chronic inflammatory responses (Weiss, 1989). In this case, the local complement activation contributes to the subsequently influx of neutrophils and macrophages. The local activation of phagocytosis leads to the release of inflammatory mediators such as lysosomal enzymes and reactive oxygen species, which are involved in mediating the tissue damage.

Pharmacological glucocorticoids are powerful inhibitors of the inflammatory response at many levels, including leukocyte trafficking and function. Most of the effects of glucocorticoids on neutrophils are inhibitory by suppressing neutrophil migration and ac-

tivation. Therapeutically, this can be counter-productive, as one of the most notable side effects of high-dose steroids is decreased resistance to infection due to impaired neutrophil bactericidal activities (Goulding et al., 1998). For example, the treatment of lupus patients is mainly based on the use of immunosuppressive drugs such as corticoids, which significantly increase the risk of infections. Furthermore, infection is a major cause of morbidity and mortality in these patients (Iliopoulos and Tsokos, 1996).

Most studies on the effects of glucocorticoids on leukocyte function are limited to high-dose therapy. In the present study, we investigated whether the oxidative burst in neutrophils mediated by complement receptors is affected by low-dose prednisone. We observed that treatment with low doses of prednisone did not affect the oxidative burst mediated by complement receptors of rat neutrophil phagocytosis. No significant differences could be observed between control and treated rats for the CL response in all studied doses and for both periods of treatment. Probably, low-dose prednisone has no effect on the key enzyme of respiratory burst, i.e., NADPH (nicotinamide adenine dinucleotide phosphate, reduced form)-oxidase.

Hoeben et al. (1998) reported that the glucocorticoids have no adverse effects on the CL of bovine PMN leukocytes in vitro at therapeutic concentrations. Forslid and Hed (1987) could not detect any effect of hydrocortisone on the ability of the neutrophils to ingest iC3b-opsonized yeast particles when they used only phagocytes that were not preactivated.

According to Forslid and Hed (1982), hydrocortisone decreases both the IgG- and C3b-mediated interactions with human neutrophils at a concentration of 5×10^{-5} M or higher, which is within probable pharmacological concentrations used in patients. They suggest that the direct effect of corticosteroids on mature human neutrophils is primarily a surface phenomenon affecting the binding abilities of IgG and C3b receptors, which secondarily will result in decreased metabolic activation.

The CL produced by stimulated PMN in the presence of luminol depends on the formation of reactive oxygen species by the cell. The light emission occurs as a result of a series of steps, which can be described in general terms as follows (DeChatelet et al., 1982): formation of superoxide (O_2^-) followed by its dismutation into H_2O_2 which, together with Cl^- and upon the action of a myeloperoxidase, produces OCl^-. This hypochlorite (OCl^-) is capable of oxidizing luminol with the generation of an excited aminophtalate anion, which relaxes to the ground state with the emission of light. Thus, the measurement of luminol-dependent CL reflects a global effect depending on the rate of production of reactive oxygen species and on myeloperoxidase activity.

With regard to the IC, the kinetics of CL is variable depending on the form in which the IC are presented to the cells (Lucisano et al., 1998), indicating that the triggering and/or the biochemical pathways might be different. Another factor might be a different rate of binding of IC to the cells.

In our present results, although the total CL produced was not different between control and treated animals, in order to evaluate if the kinetics of CL generation could be affected, we calculated the T_{max} and CL_{max}.

T_{max} reflects the rate of binding of stimulus (opZy) to the cells. The possibility that changes induced by prednisone in complement receptor expression, as well as in the biochemical pathways involved in the oxidative burst, should be considered if T_{max} is shown to differ, even though the total CL was not affected.

CL_{max} was calculated as logarithm of the maximum CL and represents a value for a point of the curve. CL_{max} differs with the logarithm of the AUC of CL profile, which represents the total response over a time period of 10 min.

Sometimes, in CL measurements the slope of the CL is slower or faster than the controls, however, the AUCs are similar. The CL_{max} can also reflect the efficient binding of the stimulus to the cells necessary to induce the NADPH-oxidase activity.

Yu et al. (1989) reported that the decrease in phagocytic ability was more prevalent in untreated lupus patients than among patients treated with corticosteroids or other immunosuppressive agents.

The phagocyte function of lupus patients treated with small doses of prednisolone is not significantly impaired (Boghossian et al., 1984). Patients treated only with low-dose prednisone (equivalent to or less

than 10 mg/day) also have increased infection rates (Iliopoulos and Tsokos, 1996). Interestingly, this dosage did not increase the infection rate in patients with rheumatoid arthritis, and, as pharmacological data shows, this dose is inadequate to suppress the immune response (Caldwell and Furst, 1991). The observed infections of SLE patients treated with low-dose corticosteroids are apparently attributable to the primary disease.

The contrast between our results and those reported in the literature may be related to differences such as drug modality, drug concentration, in vivo and in vitro experimental conditions, routes of administration, procedures and methods, species variation, sensitivity among cell types.

It can be concluded from the present results that the use of low-dose prednisone in vivo has no adverse effects on the CL of rat neutrophil phagocytosis of complement-opZy. According to these results, it can be suggested that low-dose prednisone has no effect on the neutrophil complement receptors in mediating the oxidative burst. Moreover, these results suggest that the decreased oxidative burst of SLE neutrophils, described in our previous study (Marzocchi-Machado et al., 1998), may be partially due to dysfunction and/or decreased expression of complement receptors caused by the primary disease.

Although the treatment with 257 μg for 15 days reduced the CL to one third of control (Fig. 1B, right panel), this reduction was not significant when the statistical analysis was calculated (Fig. 2B, right panel). However, in this group the animal number was only four. In fact, if we consider this dose as a high-dose prednisone, even though there are differences between treated and control, these results could be expected. The higher doses were not included because the treatment of prednisone with 257 μg/animal/day, equivalent to 90 mg/human/day, was three times higher than the highest dose prednisone that SLE patients were taking (30 mg/day) in our previous study. Furthermore, 90 mg/human/day could be considered a high dose. Thus, only one high-dose prednisone treatment was included in our study.

Although 30 and 90 mg of prednisone cannot be considered low doses, 30 mg is not immunosuppressive. These results show that these doses, three and nine times, respectively, higher than that considered as low dose (28 μg/animal or 10 mg/adult human), do not have an effect on the CL of neutrophils.

Since there are few reports in the literature about the low-dose glucocorticoid effects on complement receptor function of neutrophils, these results may help clarify the difficulty in differentiating between chronic inflammatory disease flare-up and the side effects of drug therapy.

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