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Effects of acute and long-term diazepam administrations on neutrophil activity: a flow cytometric study

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Abstract

This study analyzed the effects of acute and long-term diazepam treatments on rat peripheral blood neutrophil activity and cortisol serum levels. Rats were acutely and long-term (21 days, once daily) treated with diazepam (10 mg/kg) or its vehicle (1.0 ml/kg). Blood was collected 1 h after treatments for flow cytometric analysis of neutrophil oxidative burst and phagocytosis. Corticosterone and diazepam concentrations were also determined. Results showed that: (1) both diazepam treatments increased lipopolysaccharide (LPS) and phorbol myristate acetate (PMA)-induced neutrophil oxidative burst; (2) the increase in oxidative burst after *Staphylococcus aureus* induction in acutely treated animals was higher than that observed after long-term treatment; (3) phagocytosis is increased by acute diazepam treatment and decreased by a long-term regimen; (4) acute, but not long-term, diazepam treatment increased corticosterone levels; (5) diazepam plasmatic levels after acute and long-term treatments were not different. These results indicate the development of tolerance to diazepam effects on corticosterone serum levels but not on neutrophil activity.

Keywords: Diazepam; Flow cytometry; Neutrophil; Oxidative burst; Peripheral benzodiazepine receptor; Corticosterone; Tolerance development

1. Introduction

Benzodiazepines are among the most frequently used and prescribed class of psychotropic drugs employed in Medicine in Brazil and worldwide due to their anxiolytic, muscle relaxant and anticonvulsant effects (Ruiz et al., 1993; BHM, 1989). These benzodiazepines effects are a consequence of their action on high-affinity receptors present in the central nervous system, namely the $GABA_A$ receptors. Nevertheless, besides the central receptors described for benzodiazepines, peripheral benzodiazepine receptors have also been identified in peripheral organs (Braestrup and Squires, 1977), endocrine steroidogenic tissues (Papadopoulos, 1993) and immune organs and cells, such as polymorphonuclear and mononuclear cells (Zavala et al., 1984; Marino et al., 2001). Subsequent research on peripheral benzodiazepine receptors showed that they markedly differ from $GABA_A$ receptors both physiologically and pharmacologically (Casellas et al., 2002).

An altered peripheral benzodiazepine receptors expression was reported on human polymorphonuclear neutrophils of patients with chronic granulomatous disease (Zavala and Lenfant, 1987). This inherited phagocyte disorder is characterized by lack of superoxide anion production leading to recurrent bacterial and fungal infections. Accordingly, treatment of mice with benzodiazepines impaired the capacity of peritoneal and spleen phagocytes to produce reactive oxygen species, Interleukin-1, Tumor necrosis factor-α (TNF-α) and Interleukin-6 (Zavala et al., 1990a). These key mediators of inflammation are macrophage derived and implicated in host defense mechanisms against pathogens and tumor cells. In this respect, diazepam treatment was reported to decrease resistance to *Mycobacterium bovis* infection in hamsters (Righi et al., 1999). An impaired host resistance to *Trichinella spiralis* was also reported in rats after diazepam treatment (Schlumpf et al., 1994). Stimulation of peripheral benzodiazepine receptors present in adrenal cells

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was shown to increase glucocorticoid production (Cavallaro et al., 1992). This hormone is known to have potent immunomodulatory properties (McEwen et al., 1997). In isolated human neutrophils, diazepam concentration-dependently increased migration and phagocytosis, effects that were inhibited by the peripheral benzodiazepine receptors antagonist PK11195 (isoquinoline carboxamide) (Marino et al., 2001). Migration and phagocytosis by neutrophils are fundamental functions that allow these cells to reach inflamed tissues and remove bacteria, damaged cells and debris. Altogether, these data strongly suggest a relevant involvement of peripheral benzodiazepine receptors with innate immune responses.

Acute diazepam treatment (10 mg/kg) decreased the volume of acute inflammatory paw edema induced by carrageenan injection in rats (Lazzarini et al., 2001). In this work, we also showed that treatment with Ro5-4864, a peripheral benzodiazepine receptors agonist, reduced values of paw edema induced by carrageenan injection, confirming previous report (Torres et al., 1999, 2000). The effects of diazepam and Ro5-4864 on paw edema induced by carrageenan injection were abrogated by prior treatment with PK11195 (Lazzarini et al., 2001). In contrast, long-term (21 days) administration of diazepam (10 mg/kg) did not modify paw edema induced by carrageenan injection (Lazzarini et al., 1996, 2003), a fact that strongly suggested the development of tolerance to its effects. In the present experiment, we decided to use a flow cytometric methodology to investigate the effects of acute and long-term (21 days) diazepam treatments on neutrophil phagocytosis and oxidative burst. To look for a possible relationship between the effects induced by diazepam treatments on neutrophil activity and on corticosterone, the serum levels of corticosterone and the plasmatic levels of diazepam were also measured after acute and long-term diazepam treatments.

2. Materials and methods

2.1. Animals

One hundred and sixteen pathogen-free male Wistar rats weighing 230–250 g were used. The animals were housed in plastic cages (41 × 36 × 16 cm, five per cage) in temperature controlled (21–23 °C) and artificially lighted rooms on a 12-h light/12-h dark cycle (lights on at 7:00 a.m.) with free access to food and water. The experiments were performed in a different room, at the same temperature as the animal colony, to which the animals were transferred and maintained in their home cages 1 day before the experiments. Animals were housed and used in accordance with the guidelines of the Committee on Care and Use of Animal Resources of the Scholl of Veterinary Medicine, University of São Paulo, that are similar to that used in the European Community.

2.2. Drugs

Diazepam (COMPAZ®, Cristalia, São Paulo, Brazil) diluted in 40% propyleneglycol Ringer's solution was acute and long-term (21 days, once daily) administered intraperitoneally (i.p.) at a dose of 10 mg/kg to experimental rats; 40% propyleneglycol Ringer's solution (1 ml/kg) was used as control. This dose of diazepam was chosen to comply with our previous experiments conducted on rats (Lazzarini et al., 1996, 2001, 2003); furthermore, long-term use of diazepam in doses 5–10 times higher them the ED_{90} was reported to produce tolerance (Zanotti et al., 1999). Also, the 10 mg/kg dose is in the same range of benzodiazepines doses reported to modify immune reactions (Zavala et al., 1990b).

2.3. Sampling and peripheral blood neutrophils

Blood was withdrawn directly from the left ventricle into lithium heparin Vacutainer tubes (Becton Dickinson) immediately after their sacrifice. Blood samples (100 μl) were directly used to analyze phagocytic activity and oxidative burst of neutrophils as described below. Blood used for corticosterone serum levels and diazepam plasmatic levels were collected using the same route, but stored in Vacutainer tubes in the presence of EDTA.

2.4. Flow cytometry

A flow cytometer (FACSCalibur®, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) interfaced with a Macintosh G4 computer was used. Data from 10,000 events was collected in list mode and analyzed in Cell Quest software (Becton Dickinson Immunocytometry Systems). Cell populations were identified based on their properties on forward scatter vs. side scatter (FSC/SSC) plots, mechanically sorted and evaluated through light microscopy after staining with Giemsa dye. Fluorescence data was collected on log scale. Green fluorescence from 2′, 7′Dichlorofluorescein diacetate (DCFH) (Molecular Probes, Eugene, OR, USA) was measured at 530 ± 30 nm (FL1 detector); red fluorescence from Propidium Iodide (PI)-labeled *Staphylococcus aureus* (Sigma, St. Louis, CA, USA) was measured at 585 ± 42 nm (FL2). Propidium iodide and DCFH fluorescence were analyzed after compensation to correct for possible crossing over between propidium iodide and DCFH signals.

2.5. Oxidative burst and phagocytosis

The substances used for triggering the oxidative burst were lipopolysaccharide (LPS/100 ng), phorbol myristate acetate (PMA/100 ng) and *S. aureus* (2.4×10^9 bacteria/ml). Briefly, 100 μl of whole blood (2×10^5 cells/100 μl) was mixed with 200 μl of DCFH-DA (0.3 mM) in PBS and 100 μl of either propidium iodide-labeled *S. aureus*, PMA or

lipopolysaccharide in different polypropylene tubes. Samples were incubated under agitation at 37 °C for 20 min. Reactions were stopped by adding 2 ml of cold EDTA solution (3 mM) in order to terminate phagocytosis. After centrifugation (250 × *g* for 10 min), erythrocytes were lysed from all samples with sterile 0.2% NaCl (2 ml per tube) for 20 s. Immediately after that, 1.6% NaCl sterile solution (2 ml) was added to each sample to restore isotonicity. Samples were then centrifuged (250 × *g* for 10 min) and the cell pellets resuspended in 1 ml of cold EDTA (3 mM) for flow cytometry. Direct measurements of mean fluorescence of green and red channels were recorded as oxidative burst and phagocytosis, respectively, as proposed elsewhere (Hasui et al., 1989).

Since neutrophils exhibited higher fluorescence in the resting state (auto-oxidation of DCFH) and a much greater response after PMA stimulation, two different neutrophil populations were circumscribed in the SSC vs. FSC cytogram after PMA treatment as reported by Raidal et al. (1998). In the present experiment, only the highly responsive neutrophils after PMA were considered to analyze diazepam effects. Quantification of phagocytosis and oxidative burst was estimated by mean propidium iodide and DCFH fluorescence/cell, respectively. Data on intensity of fluorescence for both neutrophil oxidative burst and phagocytosis were expressed in percentage of their respective controls (therefore considered as 100%).

2.6. Serum corticosterone determination

Corticosterone was determined in serum using commercially available kits (Coat-a-Count®, DPC, Los Angeles, CA, USA); this procedure is based on a solid-phase radioimmunoassay in which ^{125}I-labeled corticosterone competes for a fixed time with corticosterone present in the rat sample for antibody sites. Serum samples were assayed directly without extraction or purification. In order to decrease data variability and to avoid possible effects of stress on serum

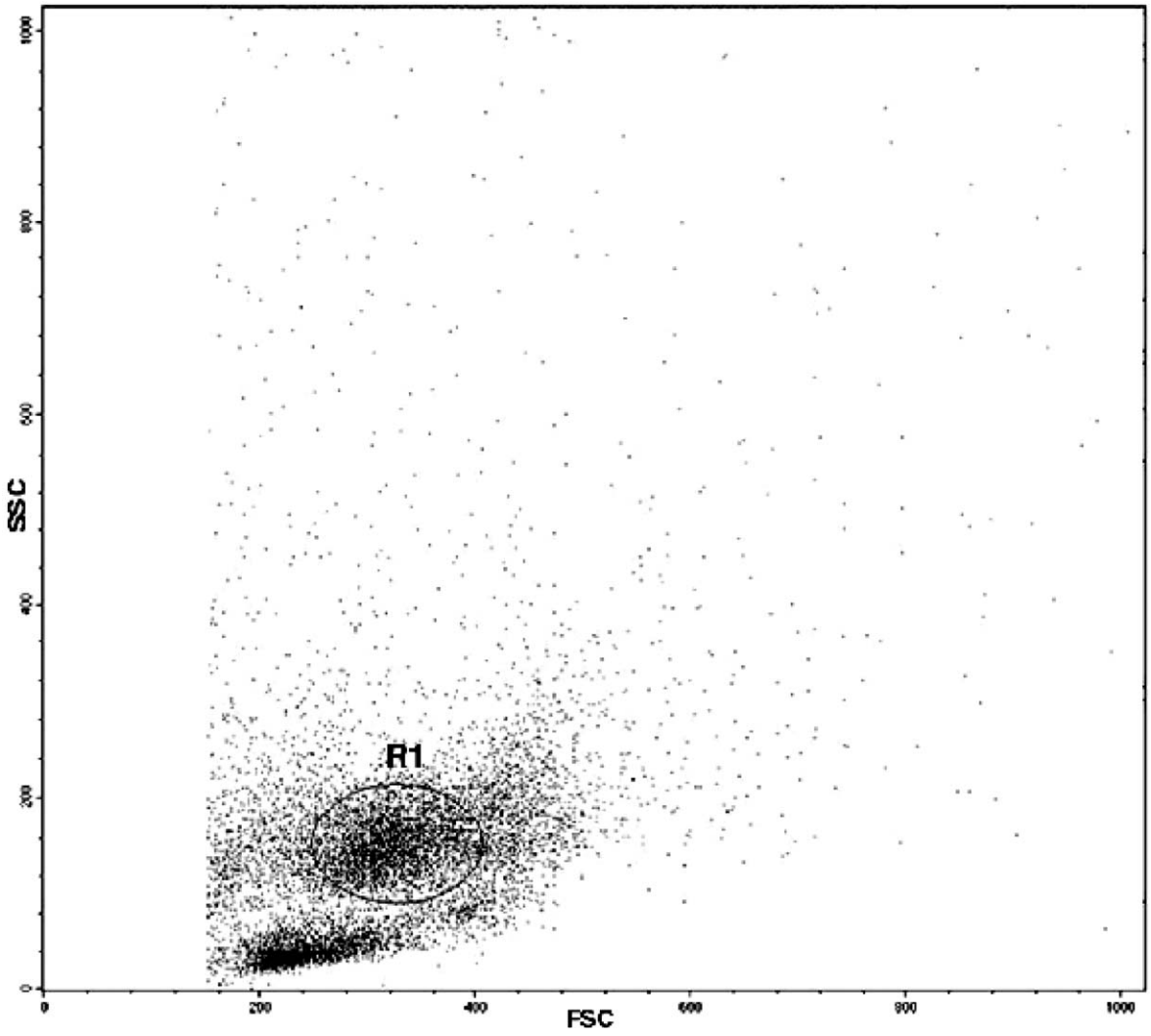

Fig. 1. Typical side scatter (SSC) and forward scatter (FSC) cytogram of rat blood leukocytes showing the R_1 gate that corresponds to neutrophils, confirmed by sorting and Giensa staining.

corticosterone levels, the rats were handled daily for habituation to the experimental conditions of blood collection, and never left alone in their home cages, which were kept far from the room where they were going to be sacrificed. In addition, rats were sacrificed at the same time of day (between 8:00 and 9:00 a.m.) in order to minimize the reported circadian variations on serum glucocorticoid levels (Albers et al., 1985).

2.7. Determination of plasma diazepam concentration

Diazepam was measured in rat plasma samples by high-performance liquid chromatography coupled to electrospray tandem mass spectrometry. Briefly, 50 ml of the internal standard solution (bromazepam 2 mg/ml in 50% methanol aqueous solution) was added to 200 ml of plasma, after which the compounds were extracted by vortex mixing during 40 s with 4 ml of a diethyl ether/*n*-hexane mixture (80:20; v/v). After centrifugation, the organic phase was separated and evaporated under a nitrogen stream at 37 °C. The dry residue was then re-dissolved with 200 ml of an aqueous acetonitrile solution (20%; v/v) containing 10 nM formic acid and 10 µl of the extract was injected into the chromatographic system. Separation of the compounds was performed on a Genesis C_{18} 4 mm analytical column (100 × 2.1 mm i.d.) isocratically eluted with an aqueous acetonitrile solution (80%; v/v) containing 10 mM formic acid delivered at a flow rate of 0.2 ml/min at room temperature. The mass spectrometer (Finnigan LCQ Deca®; Thermo Quest, CA, USA) equipped with an electrospray source was run in positive mode, and set up in multiple reaction mode (MRM), monitoring the transitions 285.1>257.1/154.0/182.0222.2 and 318.1>290.0 for diazepam and bromazepam, respectively. Under these conditions, the observed limit of detection for the method was 1 ng/ml diazepam.

2.8. Experimental design

Three experiments were done in accordance to Good Laboratory Practice Protocols (GLP) and quality assurance methods. In each experiment, two identical replications were performed. In the first, 18 animals were randomly and equally divided into two groups: E_1 and C_1. Rats from group E_1 were acutely treated with diazepam (10 mg/kg), whereas those of group C_1 received the same volume of control solution (1 ml/kg). In the 2nd experiment, 18 rats were divided into two groups E_2 and C_2 as stated above, being treated once daily, for 21 days, with diazepam (10 mg/kg) or with control solution (1 ml/kg), respectively. Blood samples were collected 1 h after acute and long-term diazepam or control solution treatments to determine both oxidative burst and phagocytosis on peripheral blood neutrophils. Finally, in the 3rd experiment, 80 rats were equally and randomly divided into four groups: E_1, E_2, C_1 and C_2. Rats from group E_1 were acutely treated with diazepam (10 mg/kg), whereas those of group E_2 were long-term treated (21 days, once daily) with similar diazepam doses. Rats from groups C_1 and C_2 received the same volume of control solution (1 ml/kg), for 1 or 21 days, respectively. One hour after the single or last diazepam (or vehicle) injection, 10

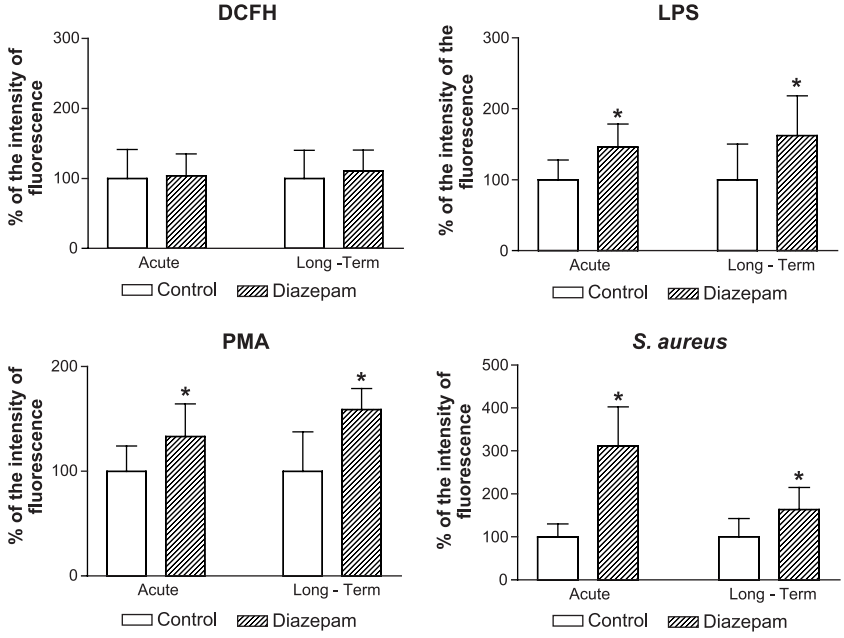

Fig. 2. Effects of acute and long-term (21 days, once daily) diazepam (10 mg/kg) treatments on neutrophil basal (DCFH), lipopolysaccharide, PMA and *S. aureus*-induced oxidative burst. Values are expressed as percentage of their respective controls, considered 100%; the mean ± S.D. of eight rats per group is presented. The values (mean ± S.D.) of rats acutely (group C_1) and long-term (group C_2) treated with control solution (1.0 ml/kg) were: 21.7 ± 8.9 and 31.1 ± 12.5 for DCFH, 58.6 ± 5.2 and 63.18 ± 15.4 for lipopolysaccharide-induced, 210.6 ± 30.7 and 301.9 ± 12.9 for PMA-induced, 81.5 ± 13.5 and 92.9 ± 12.7 for *S. aureus*-induced neutrophil oxidative burst, respectively. *$P < 0.05$ (*t*-test in relation to respective control).

rats from each group were used for determination of serum corticosterone and diazepam plasmatic levels, respectively.

2.9. Statistical analysis

Bartlet's test showed that the obtained data in all experiments were parametric ($P<0.05$). Thus, Student's *t*-test was used to analyze all data on neutrophil oxidative burst and phagocytosis. A one-way variance analysis (ANOVA) (INSTAT® 3.01 for Windows®) and Tukey test were employed to analyze corticosterone and diazepam levels. Data are presented as mean ± S.D. $P<0.05$ was considered to show significant differences for all comparisons made.

3. Results

A typical side scatter (SSC) vs. forward scatter (FSC) cytograms of rat peripheral blood leukocyte representative of those taken from rats of all groups, revealed a distinct cell population (R_1) relative to neutrophils; mechanical cell sorting showed the presence of over 98% of neutrophils in gate R_1 (Fig. 1). As expected, a heterogeneous neutrophil population composed of lower and higher responding cells was observed in blood samples taken from rats of all groups after PMA treatment. As an example, the intensities of fluorescence found in neutrophil samples retrieved from control animals (group C_1) after PMA were 167.1 ± 46.3 and 391.9 ± 50.7 for the lower and higher responsive neutrophil, respectively.

Fig. 2 shows the effects of acute and long-term diazepam treatment on neutrophil oxidative burst. An increment in green fluorescence was observed after DCFH load, allowing satisfactory measurements of diazepam effects on oxidative responses in peripheral blood neutrophils. Acute and long-term diazepam treatments did not change the intensity of basal fluorescence ($P<0.05$), i.e., the basal (DCFH) level of neutrophil oxidative burst ($C_1 = 100.0 \pm 41.3$; $E_1 = 104.2 \pm 30.9$; $C_2 = 100.0 \pm 40.2$; $E_2 = 110.7 \pm 29.9$). However, both diazepam treatments increased ($P<0.05$) the levels of fluorescence after incubation with lipopolysaccharide ($C_1 = 100.0 \pm 27.8$; $E_1 = 145.8 \pm 38.9$; $C_2 = 100.0 \pm 50.1$; $E_2 = 162.2 \pm 56.0$), PMA ($C_1 = 100.0 \pm 24.0$; $E_1 = 133.2 \pm 31.1$; $C_2 = 100.0 \pm 37.5$; $E_2 = 158.8 \pm 19.9$) and propidium iodide-labeled *S. aureus* ($C_1 = 100.0 \pm 30.0$; $E_1 = 311.3 \pm 90.8$; $C_2 = 100.0 \pm 42.8$; $E_2 = 163.3 \pm 51.7$) suggesting an increment in cellular oxidative burst followed neutrophil activation (Fig. 2). Thus, diazepam effects on oxidative burst after lipopolysaccharide, PMA and *S. aureus* stimuli were still present after long-term diazepam treatment. Furthermore, the increments in the intensity of fluorescence observed after lipopolysaccharide and PMA-induction in neutrophils retrieved from acutely treated rats were not statistically different from those detected after long-term treatments. In this respect, the increment on oxidative burst observed after incubation with *S. aureus* was smaller ($P<0.05$) after long-term diazepam treatment (group E_2) than that measured after a similar dose of diazepam given acutely (group E_1).

Data on phagocytosis of peripheral blood neutrophil are depicted in Fig. 3. An increment in red fluorescence was induced by *S. aureus* in neutrophils. This increment in fluorescence allowed satisfactory analysis of diazepam effects. Thus, acute diazepam treatment increased ($P<0.05$) not only the percentage of cells that perform phagocytosis ($C_1 = 100.0 \pm 4.9$; $E_1 = 115.9 \pm 4.7$; $C_2 = 100.0 \pm 16.0$; $E_2 = 54.8 \pm 18.8$) but also the intensity of fluorescence ($C_1 = 100.0 \pm 32.0$; $E_1 = 256.2 \pm 59.1$; $C_2 = 100.0 \pm 12.6$; $E_2 = 76.0 \pm 7.7$); this latter fact is directly related to the number of bacteria within each neutrophil. However, an opposite effect was found for long-term diazepam treatment on neutrophil activity. Indeed, both the percentage of cells performing phagocytosis and the intensity of fluorescence were decreased ($P<0.05$) after this regimen. Thus, acute diazepam treatment (10 mg/kg) increased, whereas a similar dose administered on a long-term schedule decreased neutrophil phagocytosis of *S aureus*.

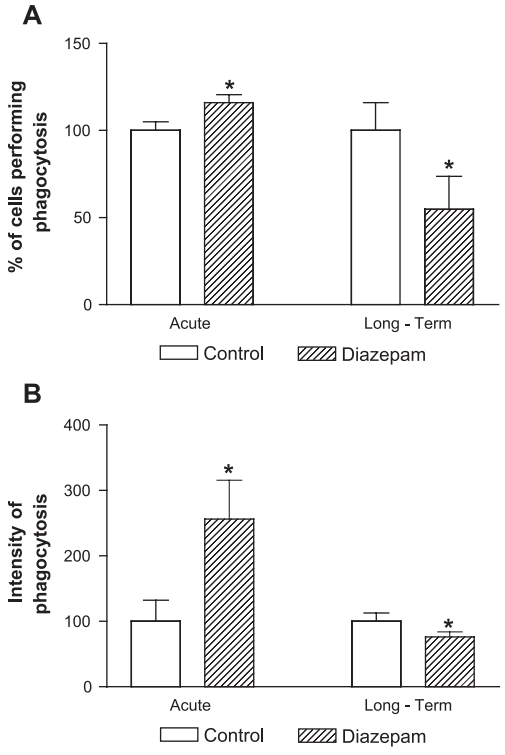

Fig. 3. Effects of acute and long-term (21 days, once daily) diazepam (10 mg/kg) treatments on the percentage of neutrophils performing phagocytosis (A) and intensity of neutrophil phagocytosis (B). Values are expressed in percentage of their respective controls, considered as 100%; the mean ± S.D. of eight rats per group is presented. The values (mean ± S.D.) of rats acutely (group C_1) and long-term (group C_2) treated with control solution (1.0 ml/kg) were: 80.5 ± 4.0 and 72.0 ± 8.4 for percentage of neutrophil phagocytosis (A), 49.1 ± 6.1 and 48.6 ± 3.6 for intensity of neutrophil phagocytosis, respectively. *$P<0.05$ (*t*-test in relation to respective control).

Table 1
Corticosterone serum levels and diazepam plasmatic levels after acute or long-term (21 days, once daily) diazepam (10 mg/kg) treatment in rats

Group	Treatment[a]	Corticosterone[b] (ng/ml)	Diazepam[b] (ng/ml)
C_1	Control acute	456.0 ± 145.5	nf [c]
E_1	Diazepam acute	1079.0 ± 322.0[d]	94.9 ± 4.63
C_2	Control long-term	676.0 ± 270.0	nf
E_2	Diazepam long-term	526.0 ± 230.0	67.0 ± 21.0

[a] Control animals received identical number of treatments and volume (1 ml/kg) of control solution.
[b] Data are mean ± S.D. of 10 animals.
[c] nf = not found.
[d] $P < 0.05$ in comparison to other groups for the same parameter (One-way ANOVA followed by Tukey test).

Table 1 shows that acute diazepam treatment (10 mg/kg) increased ($F_{(3,34)} = 11.578$; $P < 0.05$) serum levels of corticosterone in rats. Nevertheless, long-term diazepam treatment (10 mg/kg/day, for 21 days) was unable to change this hormone levels. Although somewhat higher, the corticosterone levels from group C_2 were not statistically different from those of group C_1.

The plasmatic levels of diazepam after acute and long-term 10 mg/kg treatments are also presented in Table 1. As expected, diazepam was not found in plasma of groups C_1 and C_2 animals. Furthermore, no differences were found between the diazepam levels measured 1 h after the single (group E_1) and the last long-term (group E_2) diazepam 10 mg/kg treatments.

4. Discussion

The present findings demonstrate that acute and long-term diazepam treatments changed neutrophil oxidative burst and phagocytosis in rats. Earlier studies suggested that benzodiazepines might exert stimulatory effects on immune cell functions (Ruff et al., 1985; Sacerdote et al., 1999; Marino et al., 2001) and on murine macrophage-like cell respiratory burst (Zavala and Lenfant, 1987). However, diazepam was also reported to exert inhibitory effects on human neutrophils (Goldfarb et al., 1984; Laghi-Pasini et al., 1987; Finnerty et al., 1991) and murine macrophages (Massoco and Palermo-Neto, 1999; Silva and Palermo-Neto, 1999; Righi et al., 1999). The present experiment showed that diazepam effects on neutrophil function varied according to the schedule of diazepam administration. Thus, differences, among others, in the duration of diazepam treatment seem to account at least for some of these reported discrepancies.

An increased respiratory burst, with the consequent generation of reactive oxygen species, is one of the characteristics of activated neutrophils (Yamamoto et al., 2002). In the present experiment, basal neutrophil oxidative burst was not changed by diazepam; this relevant fact suggests that neutrophil membrane activation is required for diazepam effects. In this respect, our data show that acute and long-term diazepam treatments have the same effects on lipopolysaccharide and PMA-induced neutrophil oxidative burst. However, the increment found in neutrophil oxidative burst after *S. aureus* induction was significantly higher after acute than after long-term diazepam treatment. An involvement of peripheral benzodiazepine receptors on immune cell oxidative burst has already been reported (Zavala and Lenfant, 1987). The production of oxygen derivatives is commonly known to depend on activation of NADPH-oxidase. A functional link between peripheral benzodiazepine receptors and NADPH-oxidase activation was supported by experiments showing that a monoclonal antibody recognizing peripheral benzodiazepine receptors produced a concentration-dependent stimulation of the oxidative burst (Zavala et al., 1991). Furthermore, up-regulation of peripheral benzodiazepine receptors has also been associated with maturation of cells such as HL-60, U-939 and THP1 into competent phagocytes, suggesting that these processes may be interconnected (Canat et al., 1993; Ishiguro et al., 1987). Thus, it seems reasonable to suggest that diazepam effects on lipopolysaccharide and PMA-induced increase on neutrophil oxidative burst are a consequence of peripheral benzodiazepine receptors activation followed by NADPH changes. However, the differential effects now being reported for acute and long-term diazepam treatments on PMA, lipopolysaccharide and *S. aureus*-induced oxidative burst straighten the notion that the observed increments are tied to different subcellular mechanisms. Indeed, *S. aureus*-induced oxidative burst is triggered by phagocytosis (Yamamoto et al., 2002) and, our results showed that long-term diazepam treatment decreased neutrophil phagocytosis.

Acute diazepam treatment (10 mg/kg) reduced carrageenan-induced paw edema (paw edema induced by carrageenan injection) and increased corticosterone serum levels in rats, whereas long-term diazepam treatment with a similar dose induced no changes in both parameters (Lazzarini et al., 2003). These results indicated the development of tolerance to diazepam effects on both paw edema induced by carrageenan injection and corticosterone serum levels, and suggested a relevant role for corticosterone on diazepam-induced inhibition of acute inflammation (Lazzarini et al., 2003). Thus, a direct action for diazepam on peripheral benzodiazepine receptors present on neutrophils and/or an indirect action for these benzodiazepines on peripheral benzodiazepine receptors present in steroidogenic tissues, such as the adrenals, might be related to the present results on neutrophil activity. Indeed, stimulation of the peripheral benzodiazepine receptors present on adrenal cells increases glucocorticoid production (Papadopoulos, 1993), which is consistent with the increments in corticosterone serum levels observed in this study after acute diazepam treatment, and also with that already reported by us in similar experimental context (Lazzarini et al., 2003).

However, increments on serum corticosterone levels after acute diazepam administration seems not compatible with

data presently observed on both neutrophil oxidative burst and phagocytosis. Indeed, the increment on neutrophil activity reported in the present experiment after acute diazepam treatment was also found after long-term (21 days) treatment, a moment where corticosterone serum levels were not statistically different from those measured in rats of the control group. Thus, contrary to that reported by us for diazepam effects on paw edema induced by carrageenan injection in rats (Lazzarini et al., 2003), corticosterone does not seem to play a pivotal role on neutrophil activity after diazepam treatments. It was reported that benzodiazepines do not interact uniformly with their receptors or, alternatively, that they interact with a different receptors. Indeed, It was shown not only the existence of different peripheral benzodiazepine receptors subtypes (Anholt et al., 1985; Costa and Guidotti, 1991) and their presence in different subcellular locations (O'Beirne et al., 1990; Oke et al., 1992) but also, and importantly, that peripheral benzodiazepine receptors activation results in different intracellular second messenger responses (Bisserbe et al., 1986; Schlumpf et al., 1994; Zavala et al., 1990b). In this respect, among conventional full agonists, the rate or degree of tolerance to full benzodiazepines agonists might rely on the existence of a variety of receptors (Garratt et al., 1988).

Here, we reinforce previous observation (Lazzarini et al., 2003) that long-term (21 days) diazepam treatment to rats leads to tolerance to its effects on corticosterone, an effect that suggests the occurrence of a functional adaptation of peripheral benzodiazepine receptors within adrenal gland cells. As already stated (Lazzarini et al., 2003), a simplistic pharmacokinectic explanation for the absence of effects now described for long-term diazepam treatment on corticosterone serum levels seems unlikely. Indeed, the finding that the plasmatic levels of diazepam measured 1 h after the single or the last of the repeated administrations were not statistically different from each other seems to exclude a pharmacokinectic explanation based on a fast elimination of the drug and, hence, on an insufficient exposure of peripheral benzodiazepine receptors within adrenal gland cells to diazepam. Benzodiazepine receptor binding was reported to be reduced in the cortices of mice after long-term treatment with diazepam (Zanotti et al., 1999; Natolino et al., 1996).

A pharmacokinetic mechanism is also not compatible with the reversion reported in the present experiment for diazepam effects on neutrophil phagocytosis after long-term diazepam treatment. Thus, it might be possible that long-term diazepam exposure might have led to differential changes on peripheral benzodiazepine receptors expression not only within adrenal gland cells but also in neutrophils. Pre-natal diazepam treatment induced peripheral benzodiazepine receptors down-regulation in macrophage and lymphocytes (Schlumpf et al., 1993). Peripheral benzodiazepine receptors expression in neutrophils was dramatically impaired in patients with chronic granulomatous disease, an inherited disorder due to a mutation on the gene coding for cytochrome *b*558 NADPH-oxidase component (Zavala et al., 1990b).

Long-term diazepam administration is widely employed in the treatment of anxiety; thus, the present data, although relative to a high dose of this benzodiazepines, raises concerns about the prescription of this treatment regimen to immunossupressed patients, such as HIV-positive. Indeed, neutrophil mechanisms are fundamental for the intracellular killing of bacteria and phagocytosis, also allowing cells to remove damaged tissue and debris. In addition, due to the central role played by neutrophils in host defense mechanisms, reduction of neutrophil phagocytosis after long-term diazepam treatment might also be relevant for the progression of diseases.

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