Brazilian Journal of Medical and Biological Research (1999) 32: 1145-1153
ISSN 0100-879X

Effects of diazepam on *Mycobacterium bovis*-induced infection in hamsters

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Abstract

The *in utero* exposure of hamsters to low doses of diazepam results in impaired host defense against *Mycobacterium bovis* during adulthood. Delayed developmental immunotoxicity, however, represents a specific situation that might not be general. The present experiment was undertaken to investigate the effects of diazepam on hamster resistance to *M. bovis* using adult animals. The effects of diazepam treatment on serum cortisol levels were also studied. Adult hamsters (N = 10 for each group) were treated with diazepam (E_1 = 1.0, E_2 = 2.0 or E_3 = 3.0 mg kg^{-1} day^{-1} subcutaneously) or with control solution (C) for 30 days. Seven days after the beginning of the treatment, the animals received identical inoculum concentrations of *M. bovis*. Hamsters treated with the higher (2.0 and 3.0 mg kg^{-1} day^{-1}) doses of diazepam exhibited: 1) increased granuloma areas in the liver (C = 1.81 ± 1.39, E_2 = 10.29 ± 4.64 and E_3 = 15.80 ± 4.82) and lung (C = 0.54 ± 0.55, E_2 = 6.28 ± 3.85 and E_3 = 6.31 ± 3.56) and 2) increased scores of *M. bovis* colony-forming units isolated from liver (C = 2.0, E_2 = 3.0 and E_3 = 3.5), lung (C = 1.0, E_2 = 3.0 and E_3 = 3.5) and spleen (C = 1.0, E_2 = 2.5 and E_3 = 4.0). These effects were dose dependent, and were not detected or were less severe in animals treated with the lowest (1.0 mg/kg) dose of diazepam as well as in those of the control group. Furthermore, diazepam treatment (3.0 mg kg^{-1} day^{-1} for 30 days) increased (E_3 = 71.32 ± 2.99; N = 10) the serum levels of cortisol compared to control hamsters (C = 22.61 ± 2.75; N = 10). The present data, that demonstrate an impaired defense against *M. bovis* in adult hamsters treated with diazepam, were tentatively explained on the basis of a direct and/or indirect action of diazepam on the cytokine network. The effects may be related to stimulation of peripheral benzodiazepine receptor binding sites (PBR) by macrophages and/or lymphocytes, or they may be mediated by PBR stimulation of the adrenals.

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Research supported by FAPESP (Nos. 95/8862-1 and 95/05417-7) and CNPq (No. 520050/97).

Received January 14, 1999
Accepted June 15, 1999

Key words
- *Mycobacterium bovis*
- Host resistance
- Macrophages
- Diazepam
- Immune system

Introduction

Several drugs and environmental chemicals are recognized today as potential immunotoxicants (1). Benzodiazepines (BDZ) reduce anxiety and stress responses acting on high affinity receptor sites present in the central nervous system (CNS). Because of this, they are one of the most frequently prescribed classes of psychotropic drugs in Brazil, the United States and Europe and probably worldwide (2). Nevertheless, in addition to the central receptors described for BDZ, peripheral type binding sites (PBR) have been identified in the endocrine steroidogenic tissues, immune organs and cells, such as in macrophages and lymphocytes (3-5); thus, the PBR may be a possible primary target for the immunotoxic effects of BDZ.

Indeed, in spite of the fact that BDZ are known for their low toxicity, a long-lasting reduction of mitogen-stimulated secretion of macrophage-derived cytokines was described in offspring of rats that had been exposed to diazepam during pregnancy (6,7). Furthermore, a decrease in macrophage spreading and phagocytosis was reported as a result of pre- and postnatal diazepam treatment in rats (8). Finally, an impaired host defense against *Mycobacterium bovis* in hamsters after *in utero* exposure to a dosage of 1.5 mg/kg of diazepam was also reported (9). It seems relevant to point out that alterations in the cellular immune responses induced by prenatal diazepam treatment in rats were accompanied by a reduction in the number of PBR on splenic macrophages (7) and by changes in the affinity of a splenic cell preparation which contained mainly lymphocytes (10). Delayed developmental immunotoxicity, however, is not well understood and may be a situation that cannot be generalized in terms of age. Indeed, it consists of an unknown primary effect of a chemical during early ontogeny of the immune system and a recognized functional deficit observed later in life, in the complete absence of the drug or chemical. Thus, the effects of diazepam on immune function during early life might differ from those produced in adult organisms both in a qualitative and quantitative way.

Since alterations of the immune function are considered to be toxicologically relevant when resulting in significant pathological, microbiological and/or clinical symptoms, it seemed appropriate to reconsider the immunotoxic effects of diazepam on *M. bovis*-induced infection in hamsters, now using adult animals, i.e., animals not prenatally exposed to the drug. The golden hamster is commonly used in studies on immunologic defense against mycobacteria; furthermore, the morphological substrate for tuberculosis is granulomatous inflammation where macrophages are the basic architectural and functional units (11-13). Finally, the major protective immune response against mycobacteria is cell-mediated immunity that involves killing of phagocytosed microbes as a result of macrophage activation, and lysis of the infected cells (14). The present study was therefore undertaken to analyze the effects of diazepam on host resistance to *M. bovis* infection in adult hamsters.

Material and Methods

Animals

Sixty genetically similar male golden hamsters from our colony, weighing 90-120 g and about 75 days of age, were used. The animals were housed 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 experiment. Animals were housed and used in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources of the

School of Veterinary Medicine, University of São Paulo; these guidelines are similar to those of the National Research Council, USA. Each animal was used only once.

Drugs

Diazepam (Cristalia do Brasil S/A, Itapira, SP, Brazil) was administered subcutaneously (*sc*) to experimental hamsters at doses of 1.0-3.0 mg/kg. The doses of diazepam were in the same range as those used in a prenatal experiment conducted on hamsters, rats and mice (8,9,15). Furthermore, in a study conducted on rats using a daily dose of 1.25 mg/kg of diazepam, it was found that plasma concentration of diazepam in the animals was comparable to the level seen in humans after oral or parenteral dosages (16). Some relevant data related to diazepam treatment were obtained from pilot studies conducted on hamsters and rats. Thus, it was found that the sleeping times recorded after equal diazepam doses in hamsters and rats were statistically similar; furthermore, the hypnotic dose 50% (HD_{50}) calculated for diazepam in hamsters was 7.63 mg/kg (6.81-8.78), a value not different from that obtained in rats, i.e., 7.96 mg/kg (6.85-8.98). Forty percent propylene glycol in Ringer's solution was used as the diazepam vehicle and as the control solution.

Inoculum preparation and bacteriological analysis

Mycobacterium bovis (strain AN5) activation was performed by intraperitoneal (*ip*) inoculation in hamsters before the beginning of the experiment as described by Pinheiro et al. (17). Briefly, 0.5 ml of an infectious suspension of *M. bovis* was injected *ip* into 5 hamsters; 32 days later, the animals were sacrificed, and the mycobacteria were reisolated from a pool of tissues (liver, lung and spleen). An infectious suspension of this activated strain (0.05 g tissue/25 ml 0.9% NaCl) was then used to inoculate the animals of the present experiment.

Bacteriological analysis was performed by the method of Pinheiro et al. (17). Briefly, tissue samples were removed and subjected to 3 consecutive washings in sterile physiologic saline solution (0.9% NaCl), being subsequently weighed, ground and diluted 1:10 (weight/volume) with the same saline solution and filtered. This ten-fold filtrate dilution was decontaminated by the Petroff technique, as described elsewhere (17), and centrifuged at 2500 rpm for 20 min. The pellet was resuspended in 2 ml of 0.9% NaCl solution and inoculated into 2 Petragnani culture media (5 ml); the volume of the inoculum was 0.15 ml per tube. After 34 days of incubation at 37°C, the colonies obtained were scored. The grading system employed was as follows: 0 = absence of *M. bovis* colony-forming units (CFU), 1 = below 50 CFU, 2 = from 51 to 100 CFU, 3 = from 101 to 200 CFU, 4 = from 201 to 300 CFU, 5 = from 301 to 400 CFU, 6 = from 401 to 500 CFU, 7 = more than 500 CFU.

Morphometric evaluation

Histological sections (5 µm) from representative fragments of tissues were prepared and stained with hematoxylin-eosin or by the Ziehl-Nielsen method for microscopic observation. A computerized system (Bioscan 4.0 Optimas, Edmonds, WA, USA) was used to measure the areas of the slices analyzed and the mean areas of the granulomas observed within each slice, in order to quantify the percentage of the area occupied by granulomas in each tissue.

Experimental design

Two experiments were done. In the first, 40 hamsters were divided at random into 4 groups of 10 animals each: E_1, E_2, E_3 and C. Animals of groups E_1, E_2 and E_3 were treated for 30 days with 1.0, 2.0 and 3.0 mg kg^{-1}

day^{-1} of diazepam, respectively, whereas those of group C received the control vehicle (1.0 ml kg^{-1} day^{-1}) for the same period of time. Seven days after the beginning of the treatment, all animals were injected *ip* with 0.5 ml of the activated suspension of *M. bovis*.

Beginning on the 1st day after the 1st diazepam or the control vehicle treatment, animals were examined twice daily for behavior and mortality and were weighed 1 h before and 23 days after *M. bovis* injection. The animals which died during the study were subsequently submitted to autopsy and histopathological evaluation. Animals which survived were anesthetized and sacrificed 23 days after *M. bovis* inoculation, and their livers and lungs were removed and prepared for histopathological examination. Thus, the right medial hepatic lobe and the left pulmonary lobe were removed from each hamster for morphometric evaluation. Finally, some standardized samples of the lung, liver and spleen from each animal in the four groups were also removed and used for bacteriological analysis.

In the 2nd experiment 20 hamsters were divided at random into 2 groups of 10 animals each: E and C. Animals of group E were treated with diazepam (3.0 mg kg^{-1} day^{-1}) for 30 days, whereas those of group C received the same volume and number (1.0 ml kg^{-1} day^{-1}) of the control solution injections. Immediately after the last diazepam or control solution treatment, all animals were sacrificed by decapitation and blood was collected for the determination of serum cortisol levels. Cortisol (hydrocortisone) is the most abundant circulating steroid secreted by hamsters, being considered a good indicator of adrenocortical function in this animal species (18). Cortisol was determined using commercial kits (Coat-A-Count, Los Angeles, CA, USA); this procedure is based on a solid-phase radioimmunoassay, in which ^{125}I-labeled cortisol competes for a fixed time with cortisol in the hamster sample for antibody sites. Serum samples are assayed directly, without extraction or purification. In order to decrease data variability and to avoid possible effects of stress on serum cortisol levels, the hamsters were handled daily for habituation to the experimental conditions of blood collection, and never left alone in their home cages, that were kept far from the room where animals were sacrificed. In addition, animals were sacrificed at the same time of day (between 8:00 and 9:00 a.m.) in order to minimize the reported circadian effects on serum cortisol levels (19).

Statistical analysis

Bartlett's test (20) showed that the data concerning hamster weight and serum cortisol levels were parametric ($P<0.05$). Thus, two-way (treatment x days) analysis of variance followed by the *t*-test for comparison of cell means and the *t*-test, respectively, were used to analyze these data. Number of CFU and granuloma data were analyzed by Kruskal-Wallis (KW) analysis of variance for nonparametric data, followed by the Dunnett test for multiple comparisons. Data on mortality were transformed into percentage, and analyzed by the Fisher test. The StatPac Statistic Analysis Package was used throughout with the level of significance set at $P<0.05$ for all comparisons.

Results

Table 1 shows the mean body weights of the hamsters from the different groups obtained 1 h before *M. bovis* inoculation and 23 days after inoculation, i.e., immediately before sacrifice. Thus, although *M. bovis* inoculation decreased the mean body weights of the hamsters, no statistically significant differences were found in the mean body weights of the animals of the 4 groups before and after the treatments ($F_{1,3,34} = 0.395$, $P>0.05$).

Mortality was somewhat higher in ham-

sters treated with 3.0 mg kg^{-1} day^{-1} of diazepam (C = 0%, E_1 = 0%, E_2 = 0% and E_3 = 20%) but the difference was not statistically significant.

The gross pathological alterations observed in the animals of groups C, E_1, E_2 and E_3 sacrificed 23 days after *M. bovis* inoculation consisted mainly of splenomegaly, yellowish focal point lesions on the surface of the spleen, kidney, liver, lung, mesentery and peritoneum, and adhesions in the abdominal cavities. Furthermore, histopathological examination of the tissues with yellowish focal point lesions showed the presence of multiple typical granulomas in different stages of development. A focal collection of mononuclear cells surrounded by connective tissue was observed. Some of the granulomas were characterized by the concentric appearance of the whole lesion and by the presence of aggregates of mononuclear cells, without necrosis in their central portions, while other granulomas showed the typical presence of epithelioid and giant cells, with areas of necrosis. The Ziehl-Nielsen staining method revealed the presence of several *M. bovis* bacilli within macrophages and epithelioid and giant cells in tissues from animals of all groups.

Table 2 shows the mean areas of the examined slices and granulomas induced by *M. bovis* inoculation, as well as the percentage of the area occupied by these granulomas within each tissue slice obtained from animals of groups C, E_1, E_2 and E_3. Treatment of hamsters with 2.0 or 3.0 mg kg^{-1} day^{-1} diazepam for 30 days increased the percentage of granuloma areas in the liver ($KW_{3,34}$ = 23.26, P<0.001) and lung ($KW_{3,34}$ = 24.72, P<0.05); the granuloma areas of animals in the 1.0 mg kg^{-1} day^{-1} diazepam group (E_1) did not differ significantly when compared to controls. Finally, although the areas of the granulomas were larger in the hamsters of groups E_2 and E_3, exposed to diazepam (2.0 or 3.0 mg kg^{-1} day^{-1}), no differences were detected in the morphological characteristics of these processes among the animals of the 4 groups.

The median CFU scores detected in the liver, lung and spleen of hamsters inoculated with *M. bovis* and exposed to diazepam are presented in Figure 1. The higher doses of diazepam used (2.0 and 3.0 mg kg^{-1} day^{-1}) increased the CFU scores of the hamsters

Table 1 - Body weight of hamsters injected with 1.0 (E_1), 2.0 (E_2) or 3.0 (E_3) mg kg^{-1} day^{-1} of diazepam or with 1.0 ml kg^{-1} day^{-1} of control vehicle solution (C) for 30 days, measured 1 h before and 23 days after inoculation with 0.5 ml of an *M. Bovis* (strain AN-5) suspension.

M. Bovis inoculation was performed 7 days after the beginning of diazepam or vehicle control solution treatments. Data are reported as means ± SEM for 10 animals per group. *P<0.05 compared to the same group before *M. bovis* inoculation (two-way ANOVA followed by the *t*-test).

	Body weight			
	C	E_1	E_2	E_3
1 h before *M. Bovis* inoculation	104.88 ± 5.72	111.57 ± 11.04	107.90 ± 12.78	119.60 ± 13.9
23 days after *M. Bovis* inoculation	77.28 ± 4.59*	87.06 ± 2.88*	75.53 ± 5.61*	84.93 ± 1.60*

Table 2 - Area occupied by granulomas in the liver, lung and spleen of hamsters treated with 1.0 (E_1), 2.0 (E_2) or 3.0 (E_3) mg kg^{-1} day^{-1} of diazepam or with 1.0 ml kg^{-1} day^{-1} of control vehicle solution (C) for 30 days and inoculated with 0.5 ml of an *M. bovis* (strain AN-5) suspension.

M. bovis inoculation was performed 7 days after the beginning of diazepam or vehicle control solution treatments. Data are reported as means ± SEM of percent area for 10 animals per group. *P<0.05 compared to group C (Kruskal-Wallis analysis of variance followed by the Dunnett test).

Tissue	Groups	Area (mm)		% of area occupied by granulomas
		Slices	Granulomas	
Liver	C	44.37 ± 12.83	1.81 ± 1.39	3.36 ± 2.92
	E_1	41.68 ± 11.68	4.43 ± 6.77	10.51 ± 16.49
	E_2	42.73 ± 9.32	10.29 ± 4.64*	24.07 ± 9.11*
	E_3	44.65 ± 6.01	15.80 ± 4.82*	35.72 ± 3.41*
Lung	C	24.12 ± 13.42	0.54 ± 0.55	1.84 ± 1.97
	E_1	30.77 ± 11.55	2.66 ± 3.48	11.85 ± 19.49
	E_2	24.20 ± 9.93	6.28 ± 3.85*	24.89 ± 8.57*
	E_3	16.36 ± 5.24	6.31 ± 3.56*	35.93 ± 10.80*

inoculated with *M. bovis* for the lung ($KW_{3,34}$ = 68.78, $P<0.05$), liver ($KW_{3,34}$ = 16.37, $P<0.05$) and spleen ($KW_{3,34}$ = 27.35, $P<0.05$). As can be seen, treatment of hamsters with 1.0 mg kg^{-1} day^{-1} of diazepam for 30 days did not change the CFU scores obtained.

Biochemical analysis of serum cortisol showed that diazepam (3.0 mg kg^{-1} day^{-1}) treatment of hamsters for 30 days increased ($P<0.05$) the serum levels of this hormone in relation to those measured in animals of the control group (C = 22.61 ± 2.75 and E = 71.32 ± 2.99, respectively).

Discussion

Tuberculosis is an example of an infection with an intracellular bacterium in which sensitivity is determined mainly by the host response (14). Thus, the present results demonstrate an impaired host resistance to *M. bovis* in adult hamsters after exposure to a daily dose of 2.0 and 3.0 mg kg^{-1} day^{-1} of diazepam for 30 days (groups E_2 and E_3). This conclusion is based on the following data observed after inoculation of these animals with *M. bovis*: 1) increased areas of granulomas measured in the liver and lung and 2) increased scores of CFU isolated from liver, lung and spleen. These effects were dose dependent; indeed, they were not detected or were less severe in animals of group E_1 treated with the lowest dose (1.0 mg/kg) of diazepam. The data reported here agree with those described previously in different contexts (9,21).

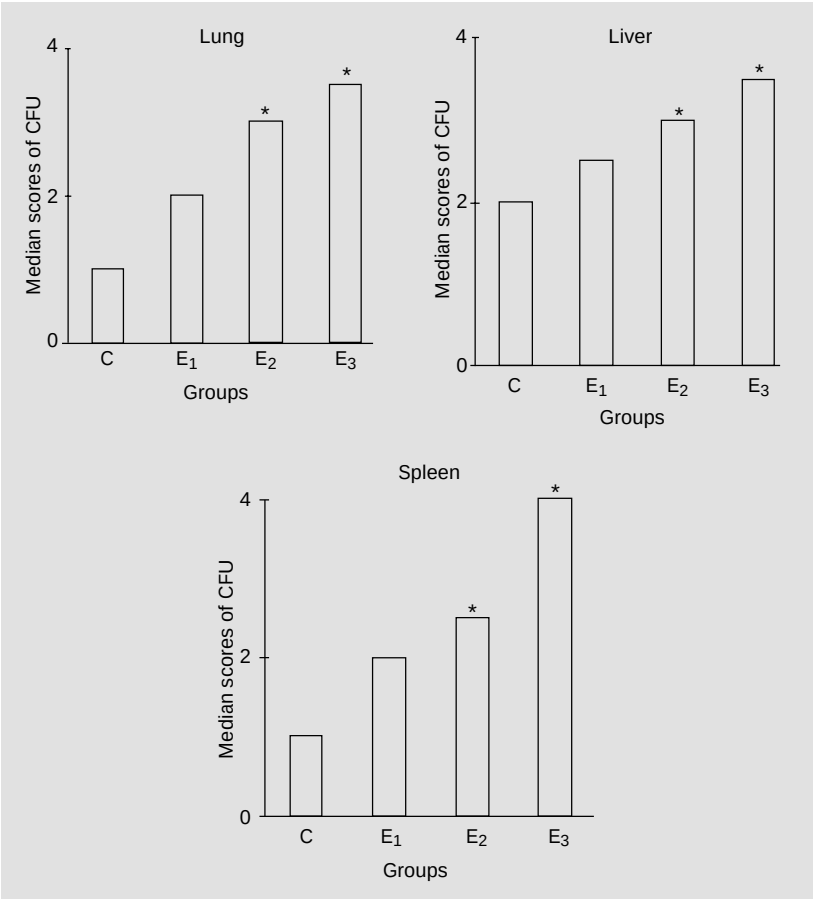

Figure 1 - Scores of *M. bovis* colony-forming units (CFU) in tissues of hamsters injected with 1.0 (E_1), 2.0 (E_2) or 3.0 (E_3) mg kg^{-1} day^{-1} of diazepam or with 1.0 ml kg^{-1} day^{-1} of control vehicle solution (group C) for 30 days and inoculated with 0.5 ml of an *M. bovis* (strain AN-5) suspension. *P<0.05, compared to control (Kruskal-Wallis analysis of variance).

The typical granulomas described in tuberculosis were observed here in the liver and lung of the hamsters of the three groups. Macrophages, epithelioid cells in palisade formation and macrophage polykaria or inflammatory giant cells and lymphocytes were detected. However, although the granulomas observed in the tissues of the hamsters of groups E_2 and E_3 were not morphologically different from each other or from those detected in control animals, the percentage of the area occupied by the granulomas was greater in diazepam-treated animals, especially in those of group E_3. Thus, prenatal diazepam treatment might have changed the modulation but not the organization of the granulomas. This may account for the decrease now detected in host resistance to *M. bovis* as indicated by the increased scores of CFU isolated from tissues of diazepam-treated hamsters.

Macrophages are the architectural and functional units of granulomas (11-13). They are phagocytic and are able to secrete a large variety of biologically active substances; they are involved in the processing and presentation of antigens to lymphoid cells and, in some way, they regulate the immune re-

sponse (22). In this respect, the ability to phagocytose and eventually digest foreign materials is the most characteristic activity of macrophages (23). According to Silva and Palermo-Neto (8), prenatal diazepam treatment (2.0 mg/kg) decreased macrophage spreading and phagocytosis, a fact also observed in adult mice acutely treated with 1.5 mg/kg of diazepam (15). Taken together, these observations might explain the results observed here about *M. bovis* resistance after diazepam treatment. Nevertheless, in spite of the fact that macrophages are the prevalent mononuclear cell population which migrates into chronic lesions, it should not be forgotten that sensitized lymphocytes also play a relevant role in these processes. Indeed, lymphocytes have been previously linked to the turnover and functional activities of macrophages in inflamed tissues (12). In this respect, it was also reported that granuloma formation is a macrophage phenomenon influenced by, but not dependent on, lymphocytes (11,12).

The mechanism of action of diazepam on macrophage/lymphocyte properties of granulomas remains unknown. Nevertheless, it might be related to cortisol production since stimulation of PBR present in steroidogenic tissues such as the adrenals has been reported to increase glucocorticoid production (3), as observed in the present study. Indeed, according to our data, diazepam treatment (3.0 mg kg^{-1} day^{-1}) for 30 days increased the serum levels of cortisol in hamsters. However, glucocorticoid hormones are known for their potent immunosuppressive and anti-inflammatory properties and thus a decrease in the extension of the granulomas and/or a change in their morphology would be expected in the present experiment. Since these events were not observed here, it seems reasonable to suggest that the effects of diazepam on granuloma modulation might have been related to other factors. In this context, the cytokine system emerges as a good candidate. Indeed, the production and release of cytokines are known to mediate both inflammatory and immune responses (24), and not only cortisol (25-27) but also diazepam through PBR stimulation of macrophage and lymphocyte membranes (6,21) have been reported to change the cytokine network.

Indeed, the inhibitory effects of glucocorticoids on the production and release of cytokines and granulocyte-macrophage colony-stimulating factor are well documented (25-27). In various experimental designs, glucocorticoids have been shown to act synergistically with exogenously added cytokines (24). In this respect, a series of experiments demonstrated that IL-1 plays a role in the initiation and development of pulmonary granulomas in mice (28). Furthermore, exposure to low doses of BDZ resulted in long-lasting alterations of the cytokine network, as indicated by the reduced release of TNF-α, IL-1, IL-6, IL-2 and interferon-γ (6,21). Evidence that IL-1 and TNF-α are involved in the phenomenon of macrophage aggregation around inert particles *in vitro* has also been shown (13), together with the participation of TNF-α in the genesis of BCG-induced granulomas in mice (29).

In the light of the present findings and discussion, it seems reasonable to suggest that PBR present in macrophages, lymphocytes and adrenals are involved in the reduced host resistance to *M. bovis* observed after diazepam treatment. Indeed, PBR and an endogenous ligand diazepam-binding inhibitor (DBI) (30) have been found to coexist in all cells of the immune and steroidogenic tissues with a significant correlation between PBR density and DBI-like immunoreactivity (31). Long-lasting changes in PBR expression on macrophage membranes (decreased B_{max}) were observed in prenatally diazepam-exposed rats (4). Finally, a relationship between PBR and modulation of oxidative reactions of neutrophils and macrophages has been described (5), a fact that might be relevant to the present findings since increased

levels of oxidative reactions were reported to occur in activated macrophages (32). Increased production of nitric oxide by activated macrophages has been reported to occur (33), an observation relevant to the present discussion. Indeed, it is known that killing of phagocytosed mycobacteria by macrophages involves nitric oxide production (34,35) and diazepam treatment has been shown to decrease macrophage activity (8,15).

Since diazepam is widely used for the treatment of anxiety, the present data raise concern about the safety of the use of this treatment in tuberculosis, mainly when patients are HIV-positive. In addition, the present findings support the idea that changes in the functional capacity of the immune system represent an important potential hazard of exposure to drugs or chemicals.

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