Effects of diazepam and stress on lung inflammatory response in OVA-sensitized rats

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Received 4 September 2001; accepted in final form 11 December 2001

The NEUROENDOCRINE AND IMMUNE systems play relevant roles in the maintenance of homeostasis. Control of immunological responses has been traditionally viewed in terms of the mutual interactions among immunocompetent cells, hormones, cytokines, and peptides. A number of recent studies are now showing that emotional states such as anxiety and stress can also influence the onset, course, and treatment of several diseases, including atopic diseases such as asthma. Studies of the emotional impact on the immune responses usually employ animal models of aversive stimulation (e.g., escapable and/or inescapable foot shocks), which allow more appropriate and complete analysis of the several endogenous variables that interact with antigen-induced inflammatory response (40–42). These approaches suggest that the ability of an organism to control a stressful situation is a critical factor in the immune response (19, 40).

Several studies conducted in the past four decades have tried to analyze the relevant interactions between emotional or psychopathological disorders and allergic and/or chronic diseases such as asthma (29, 37, 38, 52, 58). Asthma is an allergic disease characterized by an inflammation of the airways together with intermittent episodes of reversible bronchospasm (9, 21, 35). Anecdotal reports of the occurrence of stressful events before or together with the onset of a crisis in asthmatic patients are commonly heard (43). In addition, prolonged stressful situations (e.g., family conflicts) have been reported to exacerbate asthmatic crises (58). Animal models of lung anaphylactic response point in the same direction (38, 42).

Benzodiazepines (BDZ) reduce anxiety and stress responses by acting on high-affinity receptor sites present in the central nervous system (CNS). Because of this effect, they are one of the most frequently used class of psychotropic drugs worldwide (46). Nevertheless, besides the central receptors described for BDZ, peripheral-type binding sites (PBR) have also been identified for them in the endocrine steroidogenic tissues, immune organs, and in cells such as macrophages and lymphocytes (39, 48, 60).

By acting on PBR, BDZ such as diazepam have been reported to modify the cytokine network, as indicated by changes in interleukin-1 (IL-1), IL-2, IL-6, tumor necrosis factor-α, and interferon-γ (60). Probably because of this, diazepam treatment was reported to decrease macrophage spreading and phagocytosis (27) and to decrease carrageenin-induced paw edema in rats (24, 25). Stimulation of PBR present in adrenal cells was reported to increase glucocorticoid production (60), and this hor-
mone is known to have potent immunosuppressive and anti-inflammatory properties (5, 28).

The present experiment was designed to study the relationships among anxiety/stress response, diazepam treatment, and lung allergic inflammation. Specifically, ovalbumin (OVA)-sensitized rats were submitted once daily to a mild foot-shock stress and/or to diazepam treatment (2.0 mg/kg) for 14 days. Immediately after the last aversive stimulation, animals were treated with 2.0 mg/kg of diazepam [diazepam-treated and shocked (Sdz) and diazepam-treated and nonshocked (Cve) groups]. For aversive stimulation, rats were placed individually inside the shuttle box, where they received 50 escapable foot shocks (0.5 mA scrambled shocks of 5 s) through an electrified grid floor during a 30-min session. The following schedule was used: 1) a discriminative signal (S) (6 dB tone cue of 2 s) preceded the escapable foot shock; 2) each trial (tone and shock) was repeated 50 times, with an interval of 28 s between trials; 3) the set of shocks (50 trials) was repeated daily. Therefore, this shock session was repeated 13 times, beginning on sensitization day 1 and lasting until sensitization day 14. A response during the warning (S) canceled the shock delivery and terminated the S. Every shock was avoided when the responses occurred during both S and grid shocks. This discriminated-avoidance procedure has been reported to provide higher levels of acquisition and maintenance of responsiveness than the classical conditioning method (32). On sensitization day 14, Sve and Sdz animals were submitted to a single session of 50 inescapable foot shocks, discriminated by the same S and with an intertrial interval of 28 s. Animals from all groups during the first 10 min of placement inside the shuttle box were similarly placed in the shuttle box for the same period of time but without aversive stimulation.

A 3-min adaptation time in the shuttle box was provided to both control and stressed rats, i.e., the stimuli (sound or sound plus foot shock) started to be delivered 3 min after the animals were placed in the apparatus. All sessions in the operant chamber were controlled by L2T2 System software (Coulbourn, Allentown, PA) installed on an IBM-compatible computer placed in an adjacent room.

Behavioral measurements. Ultrasonic vocalizations (UV) by a rat are a known display of anxiety and/or stress levels (3). This behavioral parameter was evaluated in the present study. UV consist of many short calls, homogeneously distributed over the total duration of the shock session (53), and were recorded here, as suggested by De Vry et al. (14). For this purpose, a microphone (D 940, Pettersson Elektronik, Uppsala, Sweden) was placed in the middle of the ceiling of the shuttle box. This microphone was connected to an amplifier and to a band-pass filter that selected UV (35 dB, 20–35 kHz). The output was made visible and audible on an oscilloscope and through an earphone, respectively. A computer controlled the duration of the UV recording session; the frequency and amplitude of the UV delivered by each rat were measured with software (R900, Pettersson Elektronik). UV were detected during the adaptation period and during the first 10 min of all 14 shock sessions; recordings were made between 8:00 AM and 12:00 PM.

Experimental design. Twenty-four hours after sensitization (day 0), the rats were divided equally and at random into two experimental (Sve and Sdz) and two control (Cve and Cdz) groups. Animals of the Sdz and Cdz groups were given diazepam (2.0 mg/kg) and those of the Sve and Cve groups control solution (1.0 mg/kg) from day 1 to day 14 postsensitization. Aversive stimulation was performed from day 1 to 14 on rats of the Sve and Sdz groups. 1 h after diazepam or control solution treatment; on those days, animals of the Cve and Cdz groups were placed in the shuttle box without aversive stimulation. UV were measured daily in animals from all groups during the first 10 min of placement inside the shuttle box. All animals were submitted to an OVA challenge immediately after the discriminated inescapable foot shock (sensitization day 14), and 24 h after the OVA challenge all animals were deeply anaesthetized with chloral hydrate (10%, 3 ml/kg ip). After anesthesia, the peritoneal cavity was opened, and the animals were exsanguinated by incision of the abdominal aorta. The lungs were then imme-
diately removed and fixed in 10% formaldehyde. Sections representing peripheral areas of the lungs were obtained and processed for paraffin embedding. Histological sections of 5 µm were obtained and stained with hematoxylin and eosin.

**Morphometric analysis of peribronchial edema.** Peribronchial edema in noncartilaginous airways was measured using the point-counting technique, as previously described (50). Transversely sectioned airways were selected and examined with a light microscope (Nikon) equipped with an integrating eyepiece with 100 points and 50 lines; the airways were focused at ×1,000. The number of points of the integrating eyepieces falling on areas of edema was counted in three randomly selected areas of airway wall.

**Lung histopathological examination.** The same lungs used to determine peribronchial edema indexes were used to evaluate polymorphonucleated (PMN) and lymphomononucleated (LMN) cell infiltration. The hematoxylin-stained cells were counted under a light microscope with an integrating eyepiece and expressed as cells/unit area (10⁶ µm²).

**Passive cutaneous anaphylaxis.** Passive cutaneous anaphylaxis (PCA) reactions were produced by the technique of Mota and Wong (34). Briefly, sera from sensitized rats used in the present study were serially diluted and injected intradermally (100 µl/site) into the shaved dorsal skin of nonsensitized naive rats. After 24 h, the animals were injected intravenously with 1 ml of a solution containing 500 µg of OVA plus 2.5 mg of Evans blue in 0.9% NaCl. Thirty minutes later, the rats were deeply anesthetized with ether and killed; the skin was removed, and the diameter of the dye stain measured on the inner surface of the skin. The PCA titers represent the highest dilution of the serum that gave a dye stain of more than 5 mm in diameter.

**Data analysis.** Bartlett’s test (22) showed that the data concerning OVA-specific antibody levels were parametric (P < 0.05). Thus one-way ANOVA followed by Tukey’s test was used to analyze these data. Data on edema indexes and cellular infiltration were nonparametric and therefore were analyzed by Kruskal-Wallis (KW) ANOVA. Data on ultrasonic vocalization were transformed into percentage and analyzed by Fisher’s test. Both the Graphpad Instat Statistical package and the SigmaStat software were used, with the level of significance set at P < 0.05 for all comparisons.

**RESULTS**

**UV.** All stressed animals (Sve and Sdz groups) exhibited UV during the intertrial intervals of the discriminated foot shocks, whereas unconditioned and nonshocked rats (Cve and Cdz groups) did not display UV within the test chamber (Sve = 100%, Sdz = 100%, Cve = 0%, Cdz = 0%). Analysis of animal behavior during the first 3 min after placement inside the experimental cage revealed that UV often occurred within the period of adaptation to the shuttle box, a fact suggesting the occurrence of a conditioned emotional response (CER) in stressed rats.

**Antibody titers due to PCA.** Figure 1 shows the results obtained by PCA in naive rats that received serum from OVA-sensitized rats after the aerosol challenge. As can be seen, immune serum data did not differ between groups, i.e., both foot shock and diazepam treatment were unable to change antibody production by the rats.

**Morphometric analysis of peribronchial edema.** Photomicrographs of the airway wall of stressed and nonstressed rats are presented in Fig. 2. As expected, OVA inhalation by OVA-sensitized rats induced a peribronchial edema characterized by the presence of liquid extravasation in the space between bronchial epithelial cells and the outer limit of the airway wall in rats from the diazepam-treated groups (Sdz and Cdz). Nevertheless, the data obtained for the various groups were quite different. Indeed, as presented in Fig. 3, quantification of the OVA-induced edema showed significant differences in the data of the animals from the four groups (KW:4,17 = 106.5, P < 0.001). Thus 1) foot shock increased (P < 0.05) the edema indexes of stressed rats (Sve group) in relation to those measured in the animals of the control group (Cve and Cdz); 2) diazepam treatment decreased the indexes of edema in stressed (Sdz group) and nonstressed (Cdz group) animals in relation to those measured in animals that received control solution (Cve and Sve); 3) no differences were found between the edema indexes of rats of the Sdz and Cve groups; i.e., diazepam treatment antagonized the effect of foot shock on edema indexes.

**Lung histopathological examination.** Figure 4 depicts the number of PMN cells found in the airway wall of stressed (Sve and Sdz) and nonstressed (Cve and Cdz) rats treated or not with diazepam. No significant differences were observed (KW: 4,17, P = 0.257) in mean PMN cell numbers found around the airways of the rats of the four groups studied. On the other hand, the number of LMN cells found on the airway wall of Sve animals was significantly greater (KW:3,17 = 21.6, P < 0.001) than that found in Sdz animals and in the control groups treated or not with diazepam (Cve and Cdz). Furthermore, diazepam treatment significantly reduced the LMN cell infiltration (Sdz group) compared with that found in Sve animals (P < 0.001). No differences were found among the Cve, Cdz, and Sdz groups.
groups. Finally, no differences were found between the Cve and Cdz groups (Fig. 5).

**DISCUSSION**

Interactions between stress and immune response are undoubtedly complex, and different types of stressors might induce different immune system responses (16). In this context, stress is defined as a complex process by which an organism responds to either external environmental or psychological events that pose a challenge or danger to the organism (40). The stressful stimulus will be referred to as “stressor” and the response as the “stress response.”

The stressor employed here through the use of an escapable/inescapable foot-shock schedule triggered a UV of 20–35 kHz, which has already been referred to as an anxiogenic vocalization (13). The range of frequencies and the long duration of most of the calls reported in the present study are in accordance with data described previously (3, 31, 42). Furthermore, after the second day of training, UV also appeared during the period of habituation to the shuttle box in the complete absence of aversive stimulation. These findings strongly suggest that a Pavlovian conditioning was imposed, i.e., that the environmental context (conditioned stimulus) associated with the previously given foot shocks (unconditioned stimulus) was sufficient to induce UV (42). These calls already described as a CER by Miczek et al. (30) may be interpreted as an affective expression of a stress response (31). However, diazepam treatment did not inhibit UV emissions; most probably, the intensity of the aversive stimulation now employed was too high to be antagonized by this diaz-
Different groups (Kruskal-Wallis 1-way ANOVA). Thus at were found during the course of a stress response (5, 6, mone levels; i.e., high levels of corticosterone or cortisol positively correlated with serum glucocorticoid hor-

nance of a lung anaphylactic response (42). We observed an increase in peribronchial edema formation in the stressed group (Sve), suggesting that emotional aspects could be involved in peribronchial edema formation. The diazepam treatment reduced peribronchial edema, and this observation could be related to an effect of diazepam on the immune/inflammatory responses. This fact is in keeping with our observations that the observed changes in inflammatory responses were not a consequence of different levels of OVA-specific antibodies.

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It is common knowledge that high levels of stress are positively correlated with serum glucocorticoid hormone levels; i.e., high levels of corticosterone or cortisol were found during the course of a stress response (5, 6, 10). Thus at first sight, the increment in edema indexes observed here may seem unreliable. Indeed, glucocorticoids are largely known to have anti-inflammatory properties (54). However, the relationships among glucocorticoid hormones, stress, and IgE-mediated inflammation are not so simple, and a long-term aversive stimulation was used in the present study. There is recent experimental evidence showing that chronic mild unpredictable prenatal stress produces an increment in the allergen-induced airway inflammation in the rat offspring (36, 42).

It was now observed that diazepam treatment reduced the airway edema in both stressed (Svd) and nonstressed (Cdz) rats; thus the effects of diazepam may not be attributed to an effect on the CNS. In fact, diazepam binding to PBR found on immune cells such as macrophages (59) and lymphocytes (51) was reported to change the expression and release of several cytokines (48) and cell products (51), whose effects strongly influence the immune/inflammatory response (8, 20, 44). Furthermore, PBR are also present in adrenal cells and have been reported to increase endogenous glucocorticoid production (24, 39).

Thus our results are suggesting a relevant role for PBR in diazepam-induced changes in airway inflammation. Lazzarini et al. (24) showed that diazepam reduced carrageenin-induced paw edema and also the volume of the pleural exudate, leukocyte count, and vascular permeability in carrageenin-injected rats.

In this experiment, the number of PMN was not changed by stress or diazepam treatment. Thus it seems feasible to suppose that these cells are not involved in the changes in OVA-induced airway inflammation observed here.

We observed that diazepam treatment reduced the number of LMN cells around the airways in rats submitted to stress stimulation (Sdz group). Laitinen et al. (23) reported an increased number of lymphocytes, mainly CD4⁺ T cells, on the airway wall of mildly asthmatic patients (44). Walker et al. (55) found in humans a close relationships between T cell activation and the severity of asthmatic symptoms in humans. Glucocorticoids have been the mainstay treatment of moderate and severe persistent asthma (35) and have been known to inhibit lymphocyte/leukocyte proliferation, migration, and cytotoxicity; in vitro and in vivo studies have suggested that they suppress the secretion of certain cytokines (7, 17, 18, 45). Although glucocorticoids suppress the release of histamine by mast cells, they also act on lymphocytes, inducing a T helper 2 (Th2) response. In fact, glucocorticoid hormone activation would modulate Th1/Th2 switching and thus the intensity of the inflammatory response. This hormone also modifies the expression, production, and release of adhesion molecules and immunoglobulins (15). On the other hand, corticosterone has been shown to act synergistically with endogenous cytokines (56). Thus diazepam may directly or indirectly alter the production and release of cytokines, hence affecting the lung inflammatory response.

Diazepam treatment per se (Cdz) was unable to decrease the number of LMN cells. As described above, peribronchial edema indexes were depressed by diaze-

pam treatment alone (Cdz), which also antagonized stress-induced changes in airway edema (Sdz). Thus it seems that the effects of diazepam on edema are not directly related to those induced by this drug on LMN cell infiltration. However, caution must be exercised with this simplistic interpretation since diazepam effects on both peribronchial edema and immune cell infiltration might differ in terms of time.

The effects of diazepam on the stress-induced increment of LMN cells are not simple to explain, but cytokines seem to play a pivotal role in this observed phenomenon (4). Because diazepam per se was unable to change LMN cell number, it seems feasible to suggest the occurrence of an antagonism induced by this...
BDZ on glucocorticoids effects on the cytokine cascade and/or on Th1/Th2 switching. Indeed, diazepam and/or glucocorticoids modify cytokine expression and release (60), favoring Th1- or preventing Th2-like responses, and thus modulate the intensity of the immune/inflammatory response (57).

In the light of the present findings, it is not possible to state the effects of foot shock and/or of diazepam on Th1/Th2 responses. Nevertheless, whatever this answer might be, the present findings clearly show an effect for both stress and diazepam on lung inflammatory response, a fact that reinforces the relevance of the studies on CNS-immune system relationships.

The authors thank Dr. Benjamin Eurico Malucelli, University of São Paulo, for relevant suggestions about this experiment. This study was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo and Coordenação de Aperfeiçoamento de Pessoal do Nível Superior, to whom the authors express sincere thanks.

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