Effects of Psychological Stress and Alprazolam on Development of Oral Candidiasis in Rats

M. J. Núñez, J. Balboa, P. Riveiro, D. Liñares, P. Mañá, M. Rey-Méndez, A. Rodríguez-Cobos, J. A. Suárez-Quintanilla, L. A. García-Vallejo, and M. Freire-Garabal*

Neuroimmunology Laboratory, Department of Pharmacology, School of Medicine, University of Santiago de Compostela, 15705-Santiago de Compostela, Spain

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Psychological stress has been found to suppress cell-mediated immune responses that are important in limiting the proliferation of Candida albicans. Since anxiolytic drugs can restore cellular immunity in rodents exposed to stress conditions, we designed experiments conducted to evaluate the effects of alprazolam (1 mg/kg of body weight/day), a central benzodiazepine anxiolytic agonist, on the development of oral candidiasis in Sprague-Dawley rats exposed to a chronic auditory stressor. Animals were submitted to surgical hyposalivation in order to facilitate the establishment and persistence of C. albicans infection. Application of stress and treatment with drugs (placebo or alprazolam) were initiated 7 days before C. albicans inoculation and lasted until the end of the experiments (day 15 postinoculation). Establishment of C. albicans infection was evaluated by swabbing the inoculated oral cavity with a sterile cotton applicator on days 2 and 15 after inoculation, followed by plating on YEPD (yeast extract-peptone-dextrose) agar. Tissue injury was determined by the quantification of the number and type (normal or abnormal) of papillae on the dorsal tongue per microscopic field. A semiquantitative scale was devised to assess the degree of colonization of the epithelium by fungal hyphae. Our results show that stress exacerbates C. albicans infection of the tongues of rats. Significant increases in Candida counts, the percentage of the tongue’s surface covered with clinical lesions, the percentage of abnormal papillae, and the colonization of the epithelium by fungal hyphae were found in stressed rats compared to those found in the unstressed rats. Treatment with alprazolam significantly reversed these adverse effects of stress, showing that, besides the psychopharmacological properties of this anxiolytic drug against stress, it has consequences for Candida infection.

Candida albicans is an example of an opportunistic pathogen frequently isolated from the human mouth, yet few carriers develop clinical signs of candidiasis. The most common predisposing factors to oral candidiasis are immunosuppressive therapy, immunoincompetence, and immunodeficiencies, indicating that the host immune system provides a protective mechanism against superficial invasion by Candida.

Several lines of evidence indicate that cell-mediated immunity is important in limiting the proliferation of Candida; thus, this opportunistic human pathogen preferentially causes invasive and disseminated infections in patients with defective phagocytic defenses and serious mucocutaneous infection in patients with deficiencies in T-cell function. Phagocytes appear to protect the host from fungal colonization even in the absence of adaptive immune mechanisms, while as-yet-undefined T-cell-dependent factors seem necessary for the control of C. albicans on body surfaces (31).

In our previous research, we had observed adverse effects of stress on natural and specific immune responses that may predispose the host to more severe Candida infections (22). On the other hand, treatment with benzodiazepines (BZDs), such as alprazolam, was found to attenuate some of the effects of stress on the immune systems of rodents, such as T-cell depletion, the inhibition of the blastogenic and cytotoxic activities of spleen cells (14, 15, 18), impaired delayed type hypersensitivity (38), and defects in phagocytosis (21). We have already tested this drug in laboratory animal models of infection showing a correlation between the immunoprotective effect of alprazolam and the host resistance against bacteria (16) and viruses (19, 20). Despite other known or unknown mechanisms, central pharmacological effects regulating the release of neuroendocrine hormones, such as adrenal corticotropic hormone (ACTH), should be involved, at least in part, in the effects of alprazolam on immunocompetence. Nevertheless, there is little data on the effects of this compound on the development of fungal infection. In order to further elucidate this relationship, we studied the effects of alprazolam on the development of oral candidiasis in rats exposed to a repeated auditory stressor.

MATERIALS AND METHODS

Animals. Two-month-old male pathogen-free rats of the Sprague-Dawley strain (Interfauna Iberica, S.A., Barcelona, Spain) weighing 180 to 200 g were used. They were housed individually in filter-top cages and screened for the presence of C. albicans by plating oral swabs on YEPD (yeast extract-peptone-dextrose) agar (Sigma Chemical Co., St. Louis, Mo.) (17, 31). The cages were kept in a temperature-controlled (22 to 24°C) and humidity-controlled animal room, with an alternating light-dark cycle (lights on at 0600 and lights off at 1800) and with food (diet A.03; Panlab, Barcelona, Spain) and sterile water ad libitum.

Procedure. Following verification that the rats were free of C. albicans, they were randomly divided into six experimental groups of four animals each according to the treatment they were to be submitted to: group 1, control (i.e., no stress or placebo); group 2, unstressed rats injected with placebo; group 3, unstressed rats injected with alprazolam; group 4, stressed rats with no treatment; group 5, stressed rats injected with placebo; group 6, stressed rats injected with alprazolam.

* Corresponding author. Mailing address: Neuroimmunology Laboratory, Department of Pharmacology, School of Medicine, University of Santiago de Compostela, 15705-Santiago de Compostela, Spain. Phone: 34 600 942 256. Fax: 34 981 573 191. E-mail: ffregar@usc.es.
were analyzed by Student’s t test. *, differences between stressed and unstressed rats significant when \( P < 0.05 \); †, differences between placebo- and alprazolam-treated rats significant when \( P < 0.05 \).

**RESULTS**

*C. albicans* counts at 2 and 15 days after inoculation (Table 1), as well as the percent area of clinical lesions in the dorsal tongue (Table 2), were increased in stressed rats compared with those in unstressed animals (differences, \( P < 0.05 \)). A decrease in the total number of papillae and an increase in the percentage of abnormal (atrophic and hypertrophic) papillae (Fig. 1) were observed in stressed animals (differences, \( P < 0.01 \)). On the semiquantitative scale of colonization of the epithelium by fungal hyphae, stressed rats (Fig. 2) scored higher than untreated controls (differences, \( P < 0.05 \)). Neither placebo nor alprazolam significantly affected those parameters in unstressed rats (\( P > 0.05 \)), with the only exception that placebo increased the degree of colonization of the epithelium.

**TABLE 1. C. albicans counts from tongues of rats**

| Group              | Mean Candida count \((10^6 \text{ CFU/ml}) \pm \text{SD}^a\) |
|--------------------|----------------------------------------------------------|
|                    | Controls       | Placebo       | Alprazolam    |
| Unstressed         |               |               |               |
| Day postinoculation|               |               |               |
| 2                  | 60.75 ± 2.04  | 61.23 ± 1.90  | 60.95 ± 1.42  |
| 15                 | 4.53 ± 0.10   | 4.72 ± 0.17   | 4.95 ± 0.19   |
| Stressed           |               |               |               |
| Day postinoculation|               |               |               |
| 2                  | 66.83 ± 1.72* | 67.03 ± 1.02* | 62.16 ± 1.12*† |
| 15                 | 1.62* 15.70*  | 1.90* 11.85*  |

\( ^a \) *C. albicans* counts from the tongues of rats at times 2 and 15 after inoculation. Establishment of *C. albicans* infection was evaluated by swabbing the inoculated oral cavity with a sterile cotton applicator, followed by plating on YEPD agar. Each value is the mean ± standard deviation for four rats. Values were analyzed by Student’s t test. *, differences between stressed and unstressed rats significant when \( P < 0.05 \); †, differences between placebo- and alprazolam-treated rats significant when \( P < 0.05 \).
in unstressed animals. In contrast, treatment with alprazolam significantly ($P < 0.05$) reversed the adverse effects of stress in all parameters assayed.

Clinically evident lesions and inflammatory changes of the underlying connective tissue were observed 15 days after $C.\text{ albicans}$ inoculation. The latter were found in all experimental groups, but they were more evident in stressed rats. Animals showed macroscopic focal patchy atrophy of the dorsal tongue papillae. Light microscopy showed localized dense zones of hyphal penetration of the keratin layer in the giant conical papillae and filiform papillae of the dorsal tongue. Microabscesses in the keratin and the superficial spinous layers were observed in association with hyphal invasion. The underlying connective tissue showed a mild chronic inflammatory cell infiltrate. Those papillae that supported the Candida growth appeared shorter and blunter than the surrounding uninfected papillae.

Scanning electron microscopy (Fig. 3) of the dorsal tongues showed a higher loss of papillae in the giant conical and filiform areas of the specimens together with an increase in the size of the flat central portion of the lesion in stressed rats in comparison with unstressed animals. This adverse effect of stress was also reduced by the administration of alprazolam.

**DISCUSSION**

Our results show that stress exacerbates $C.\text{ albicans}$ infection of the tongues of rats. Significant increases in Candida counts, the percent area of clinical lesions, the percent abnormal papillae, and the colonization of the epithelium by fungal hyphae were found in stressed rats compared with those found in unstressed animals. Treatment with alprazolam partially reversed those adverse effects of stress on the development of oral candidiasis. Alprazolam was found to reverse many of the effects of stress on $C.\text{ albicans}$ infection of the tongues of rats, including Candida counts, the percent area of clinical lesions, the percent abnormal papillae, and the colonization of the epithelium by fungal hyphae.

Clinical and experimental observations indicate that the opportunistic proclivities of this fungus vary considerably, depending on the nature of the immunological defect of the victim. Patients with qualitative or quantitative defects of phagocytes are mainly prone to the invasive form of this mycosis (10, 11, 37). In contrast, defective T-cell-mediated immunity has been specifically associated with thrush and other forms of candidiasis limited to mucocutaneous surfaces (11, 12, 24, 26). Krause and Schaffner (28) demonstrated that cyclosporine, a relatively selective suppressor of T-cell-mediated immunity and NK cell activity, promoted the formation of thrushlike lesions on cyst surfaces and impeded the elimination of $C.\text{ albicans}$ from such lesions, but it had no effect on systemic candidiasis induced by intravenous inoculation.

Our results are in line with the previous literature on the stress-induced modulation of the immune system. Changes in murine splenic cytotoxic activities, mediated by NK cells and

![FIG. 1. Percentage of normal papillae in the tongues of rats. The results are the means ± standard deviations of four animals. Values were analyzed by using Student's $t$ test. *, differences between stressed and unstressed rats significant at $P < 0.05$; #, differences between placebo and alprazolam significant at $P < 0.05$.](image-url)
cytotoxic T lymphocytes have been reported (10–12, 24, 26, 32, 37). Stress also interferes with the activity of phagocytosis and T-cell-dependent antibody responses (21, 28).

The mechanism by which stress inhibits the cellular immune response has been widely studied. A molecular basis for bidirectional communication between the immune and neuroendocrine systems has been described previously (5). Cell-to-cell communication between the immune and the neuroendocrine systems is primarily mediated by hormones and neuropeptides that reach lymphoid organs and cells through the vascular system or directly through the autonomic connections between nerve endings and lymphoid organs (1, 8). Receptor sites are present in lymphoid cells for many hormones and neurotransmitters (6, 39). A number of molecules produced by cells of the nervous system such as ACTH, PRL, opioid peptides, GH, TSH, dynorphin, dopamine, and others have been shown to have the ability to modulate immune functions.

On the other hand, humoral factors generated by the immune system, such as thymic peptides and lymphokines, modulate neuroendocrine functions. In addition, in the course of lymphocyte activation, lymphoid cells may produce hormonal substances identical to those produced by the hypophysis, such as ACTH, TSH, GH, PRL, gonadotrophin, and β-endorphin (6).

At least one of the neuroendocrine responses to stress, such as the rise in plasma corticosterone concentrations via ACTH secretion, has an easily demonstrable destructive effect on specific cells and tissues that are required for optimal immune defense (4, 34). In our previous studies, we observed a stress-induced increase in ACTH levels proportional to the decrease in T-cell populations (15). Nevertheless, in these studies, we observed that adrenalectomized mice showed a lower pattern of immunosuppression in comparison with sham-operated mice. So, this led us to believe that other neuropeptides and neurotransmitters could be involved in the immunosuppressive response to stress.

The effects of alprazolam, an anxiolytic drug with high affinity for central BZD receptors on the pathogenicity of this opportunistic fungus could be attributed, at least in part, to its well-known protective effects against the immunosuppressive response to the type of stress assayed here. The recovery of the immune state of the victim could decrease the pathogenicity of this opportunistic fungus. In this regard, in our previous studies, we demonstrated that alprazolam reversed the suppressive

FIG. 2. Semiquantitative scale to assess degree of colonization of epithelium by fungal hyphae. In this scale, the absence of colonization was given a score of 0, while maximal colonization, in which an excess of 50 hyphae could be seen in each high-power field (×400), was assigned a score of 4. The other scores were 1 for 1 to 5 hyphae, 2 for 6 to 15 hyphae, and 3 for 16 to 50 hyphae. Each of the four shaded areas on each bar indicates one animal. Wilcoxon signed rank sum test for paired comparisons and the Kruskal-Wallis test for multiple comparisons were used. \( * \), differences between controls (C) and placebo-treated unstressed rats (P) significant at \( P < 0.05 \); \( # \), differences between controls and stressed rats (S) with no treatment significant at \( P < 0.01 \); \( \bullet \), differences between unstressed and stressed rats injected with placebo (S+P) significant at \( P < 0.01 \); \( \dag \), differences between placebo-treated and alprazolam-treated (A) unstressed animals significant at \( P < 0.05 \); \( \circ \), differences between placebo-treated and alprazolam-stressed animals (S+A) significant at \( P < 0.01 \).
effects of stress on the activity of phagocytosis, T-cell populations, the blastogenic response of spleen cells, murine splenic cytotoxic activities, mediated by NK cells and CTL. Fride et al. (23) found that low doses (0.02 to 1.0 mg/kg) of alprazolam significantly increased the NK cell activity, mixed leukocyte reactivity, and mitogen-induced lymphocyte proliferation in unstressed mice.

The mechanism of action of BZDs on the immune system remains to be defined. A dual approach has been described at the present time. First, central pharmacological effects related to the central type BZD receptors that facilitate inhibitory GABA neurotransmission in the central nervous system may regulate the release of neuroendocrine hormones involved in the immune response to stress. The ability of alprazolam to decrease the stress-induced increase of ACTH levels (29) has been demonstrated to play a important role in the immunoprotective effects of this drug. Nevertheless, significant immunoenhancing effects of alprazolam were also appreciated in stressed adrenalectomized rats (15), suggesting that the modulatory effect of this BZD agonist on other neurohormones like opioid peptides, PRL, melatonin, TSH, or GH could also be involved (13).

A second aspect of the effects of BZDs is the existence of a BZD receptor with high affinity on immune cells that express the so-called peripheral specificity for BZDs (41). Nevertheless, alprazolam is described in the literature as strict central type ligand of the BZD receptor (29). Alprazolam has potent PAF antagonist properties (27) that seem to affect T-cell, B-cell, and macrophage responses under in vitro conditions (9).

One could ask whether secondary (nonimmune or biochemical) effects of the drug treatment might account for the final observations and whether or not stress might break down the state of tolerance normally associated with Candida infection (as opposed to acting solely as an immunopotentiator). Although these considerations should be taken into account, our previous data concerning the immunomodulatory effects of alprazolam under stress conditions (14, 15, 18, 21, 38) lead us to consider immune changes as the main factor involved on the effects of stress and alprazolam on the evolution of oral candidiasis in rats.

A second question concerns the biological significance of our results. Although our data at present show stress may leave the subject vulnerable to the action of C. albicans and provide evidence of a protective effect of alprazolam on the development of oral candidiasis in rats, the biological significance and health relatedness of these findings should be assessed. In this respect, differences between untreated stressed rats and placebo- or alprazolam-stressed rats are statistically significant, but in some parameters, they are not striking. Moreover, there is a relationship between differences obtained in different determinations, but there is not a mathematical correlation as expected.

The large number of interactions at molecular, cellular, and functional levels between the nervous system and the immune system characterizing the operational compositions and expressions of the neuroimmune network make complex isolation of the pathways in which stress and alprazolam may be involved in the regulation of the host defense mechanisms against infection. Nevertheless, the literature has provided evidence that stress-induced immunosuppression and alprazolam-induced immunoprotection are in a relationship with susceptibility to bacteria (16), virus (20), and, as a conclusion of the present investigation, Candida infection.

REFERENCES

1. Adler, R., D. Felten, and N. Cohen. 1990. Interactions between the brain and the immune system. Annu. Rev. Pharmacol. Toxicol. 30:561–602.
2. Allen, C. M., G. C. Blozis, S. Rosen, and J. S. Bright. 1982. Chronic candidiasis of the rat tongue: a possible model for human median rhomboid glossitis. Dent. Res. 61:1287–1291.
3. Allen, C. M., R. Paulson, and R. Duncan. 1989. Clinical, histologic and scanning electron microscopic study of the development of chronic candidiasis of the rat tongue. J. Oral Pathol. Med. 18:352–359.
4. Andreoli, A., C. Toban, and G. Garrone. 1989. Stress depression immunity: current trends in research in the field of psychoimmunology. Ann. Med. Psychol. 147:35–46.
5. Blalock, J. E. 1990. Molecular mechanisms of bidirectional communication between the immune and endocrine systems. Int. J. Neurosci. 51:363–364.
6. Blalock, J. E., K. L. Bost, and E. M. Smith. 1985. Neuroendocrine peptide hormones and their receptors in the immune system: production and action. J. Neuroimmunol. 10:31–40.
7. Bowen, W. H., S. K. Pearson, and D. A. Young. 1988. The effect of desalination on coronal and root surface caries in rats. J. Dent. Res. 67:21–23.
8. Bullock, K. 1987. The innervation of immune system tissues and organs. p. 33–47. In C. W. Cotman, R. E. Britton, A. Galaburda, and B. McEwen (ed.), The neuro-immune-endocrine connection. Raven Press, New York, N.Y.
9. Chang, M. P., S. C. Castle, and D. C. Norman. 1991. Suppressive effects of alprazolam on the immune response of mice. Int. J. Immunopharmacol. 13:259–266.
10. Cohen, M. S., R. E. Isturiz, H. L. Malech, R. K. Root, C. M. Wilfert, L. Gutman, and R. H. Buckley. 1981. Fungal infection in chronic granulomatous disease. Am. J. Med. 71:59–66.
11. Edwards, J. E., Jr. 1978. Severe Candida infections: clinical perspectives, immune defense mechanisms, and current concept of therapy. Ann. Intern. Med. 89:91–106.
12. Edwards, J. E., Jr. 1986. Moniliasis of the skin, p. 50–67. In A. I. Braude (ed.), Infectious diseases and medical microbiology. Saunders Company, Philadelphia, Pa.
13. Faith, R. E., A. J. Murog, and N. P. Plotnikoff. 1991. Interactions between the immune system and the nervous system. p. 287–303. In N. Plotnikoff, A.
