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Effects of Psychological Stress and Fluoxetine on Development of Oral Candidiasis in Rats▽

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Psychological stress has been found to suppress cell-mediated immune responses that are important for limiting the proliferation of *Candida albicans*. Fluoxetine has been observed to reduce negative consequences of stress on the immune system in experimental and clinical models, but there are no data on its effects on oral candidiasis. We designed experiments to evaluate the effects of fluoxetine 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. Stress application and treatment with drugs (placebo or fluoxetine) were initiated 7 days before *C. albicans* inoculation and lasted until the end of the experiments, on day 15 postinoculation. Establishment of *C. albicans* infection was evaluated on days 2 and 15 after inoculation. 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 showed that stress exacerbates *C. albicans* infection in 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 hyphae were found in stressed rats compared to the nonstressed ones. Treatment with fluoxetine significantly reversed these adverse effects of stress. Besides the psychopharmacological properties of fluoxetine 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 immunosuppresive therapy, immunoincompetence, and immunodeficiencies, indicating that the host immune system provides a protective mechanism(s) 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 infections 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 (29).

Previous research demonstrated adverse effects of stress on natural and specific immune responses (1, 4, 9, 18, 21) that might predispose the host to more severe *Candida* infections. On the other hand, treatment with fluoxetine, a nontricyclic antidepressant drug, was found to attenuate some effects of stress on the immune systems of rodents, such as T-cell depletion, the inhibition of the blastogenic (15) and cytotoxic activities of spleen cells (34), and defects in phagocytosis (17). Nevertheless, there are few 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 fluoxetine 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 yeast extract-peptone-dextrose agar (YEPD; Sigma Chemical Co., St. Louis, MO) (16, 29). 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*. All experimental protocols adhered to the guidelines of the Animal Welfare Committee of the Universidad of Santiago de Compostela. In addition, all efforts were made to minimize animal suffering and to reduce the number of animals used.

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 to which they were to be submitted: group 1, control (i.e., no stress or placebo); group 2, nonstressed rats injected with placebo; group 3, nonstressed rats injected with fluoxetine; group 4, stressed rats with no treatment; group 5, stressed rats injected with placebo; group 6, stressed rats injected with fluoxetine.

Stress procedure. Noise was produced by a loudspeaker (15 W), installed at a distance of 30 cm above the cage and driven by a white noise generator emitting all the frequencies in the range 0 to 20 kHz. A precision sound-level meter was used to set the intensity of sound to 100 dB uniformly in the cage. The rats were subjected to a broad band noise at 100 db daily for 5 s every minute during (at random) either a 1- or 3-hour period around midnight, at the height of the diurnal activity cycle (32). All stressed rats were subjected to the same stress schedule. Nonstimulated rats were exposed only to the normal activity of the animal room. Stress application started 7 days before *C. albicans* inoculation and lasted until the end of experiments, on day 15 postinoculation.

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TABLE 1. *Candida albicans* counts from the tongues of rats

Test group and time postinoculation	Mean cell count[a] (10^4 CFU/ml) ± SD		
	Controls	Placebo	Fluoxetine
Nonstressed			
Day 2	59.98 ± 3.47	60.11 ± 1.82	59.68 ± 3.39
Day 15	6.1 ± 0.37	5.93 ± 0.53	5.78 ± 0.51
Stressed			
Day 2	78.95 ± 5.47	80.10 ± 6.11	71.05 ± 2.80
Day 15	7.68 ± 0.39	7.93 ± 0.33	6.43 ± 0.49

[a] *C. albicans* counts from the tongues of rats at 2 and 15 days 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 represents the mean ± SD of four rats. Values were analyzed by Mann-Whitney U test. Differences between nonstressed and stressed rats with no treatment were significant ($P < 0.05$) at days 2 and 15. Differences between nonstressed and stressed rats injected with placebo were significant ($P = 0.021$) at days 2 and 15. Differences between stressed rats with no treatment and those injected with fluoxetine and differences between stressed rats injected with placebo and those injected with fluoxetine were significant ($P < 0.05$) after 2 and 15 days of treatment. Differences between nonstressed rats with no treatment or injected with placebo and between nonstressed rats with no treatment or injected with fluoxetine were not significant ($P > 0.05$) after 2 and 15 days.

Treatment with drugs. Fluoxetine HCl was obtained as commercially available 20-mg capsules (Prozac; Lilly Co., Madrid, Spain), prepared following the technique of Brandes et al. (7) and subcutaneously injected at dosages of 5 mg/kg of body weight, in a volume of 1 ml/kg of H_2O. The same volume of diluent was used as placebo. Drugs were daily administered at 2130 during all period of stress application.

Surgical hyposalivation. As in humans, xerostomia in rats facilitates the establishment and persistence of *C. albicans* infection in the mouth; therefore, it constitutes a suitable animal model for the study of oral candidiasis (22). Sialoadenectomy in rats causes intense xerostomia, but the minor salivary glands, the main producers of mucin, an important barrier for mucosal permeability and a major source of immunoglobulin A, were preserved. In our experiment, xerostomia was surgically provoked in all rats 1 month before treatment with drugs, and stress applications were initiated. The rats were anesthetized with 44 mg of ketamine (Ketolar; Parke-Davis, Barcelona, Spain) per kg of body weight and 1 mg of diazepam (Valium; Roche, Madrid, Spain) per kg (44). The parotid salivary ducts of the animals were ligated, and the submandibular and sublingual salivary glands were surgically removed according to procedures previously described (6, 28, 29).

Source and culture of *C. albicans*. The *C. albicans* organisms used to inoculate the rats were obtained from a patient with erythematous oral candidiasis (16). The *Candida* strain was grown on YEPD agar plates at room temperature (38). The isolated organisms were identified as *C. albicans* by a germ tube test and chlamydospore production as described by Schaar et al. (39).

Inoculation of *C. albicans*. The *C. albicans* cells isolated were prepared for inoculation by suspending colonies in sterile buffered saline and washed twice by centrifugation before being resuspended in normal saline. The concentration of organisms was adjusted to 3×10^8/ml based on the optical density at 300 nm (OD_{300}) (3). The tongues of the animals were swabbed on two successive days with a cotton-tipped applicator saturated with 0.1 ml of fresh inoculum (29).

Quantification of *C. albicans* cells. Establishment of *C. albicans* infection was evaluated by swabbing the inoculated oral cavity with a sterile cotton applicator, followed by plating on YEPD agar (22, 29). Samples collected 2 days after inoculation and at the end of experiment were taken by the same person, who was blinded to the treatments given. The cotton applicator was immediately immersed in 0.99 ml of sterile isotonic saline to obtain a dilution of 10^{-2}, and it was agitated for 2 min. This dilution was considered to be 10^{-2}. Dilutions up to 10^{-5} (0.1 ml) were cultured in duplicate in Sabouraud's dextrose agar at 37°C for 48 h. *Candida* colonies were counted in plates exhibiting between 30 and 300 colonies. Plates with less than 30 colonies in the 10^{-2} dilution were considered to have 10^1 cells (22).

Clinical lesions. At the end of the experimental period, all animals were sacrificed by asphyxiation in a CO_2 atmosphere and were then decapitated. The dorsal tongue was photographed *in situ* at a magnification of ×10 (3). Clinical lesions were measured with a digital imaging system (Técnicas Médicas MAB, Barcelona, Spain) and expressed as the percentage of the surface area of the tongue that was covered with the lesions.

TABLE 2. Areas of clinical lesions

Test group	Mean % area of tongue with clinical lesions[a]		
	Controls	Placebo	Fluoxetine
Nonstressed	5.81 ± 0.92	6.47 ± 0.57	6.59 ± 0.49
Stressed	22.80 ± 4.8	25.11 ± 1.84	12.47 ± 1.95

[a] Percentage area of lingual candidiasis in rats after 15 days of oral inoculation. Rats were sacrificed and the dorsal tongue was photographed *in situ* at 10× magnification. Clinical lesions were measured using a digital imaging system. Results are means ± SD of four animals. Values were analyzed with an arcsin (square root) transformation, ANOVA, and Student's *t* test. Differences between nonstressed and stressed rats with no treatment were significant ($P < 0.05$; $t = 6.849$). Differences between nonstressed and stressed rats injected with placebo were significant ($P < 0.05$; $t = 19.011$). Differences between stressed rats with no treatment or those injected with fluoxetine and between stressed rats injected with placebo or fluoxetine were significant ($P < 0.01$). Differences between nonstressed rats with no treatment and those injected with placebo and between nonstressed rats with no treatment and those injected with fluoxetine were not significant ($P > 0.05$).

Tissue handling. The tongues from the rats were hemidisected in the sagittal plane, with half of the lesion immersed in 10% buffered formalin for routine processing and the other half placed in 2.5% glutaraldehyde with 0.1 M Sorensen's phosphate buffer at 4°C (3).

Light microscopy. Both hematoxylin and eosin and the periodic acid-Schiff stains were used. *C. albicans* infection was assessed by evidence of lesions and by hyphal colonization on the dorsal tongue (3, 35) detected using a digital imaging system. Tissue injury was determined by quantification of the number and type (normal, atrophic, and hypertrophic) of papillae per microscopic field (magnification, ×46). A semiquantitative scale was devised to assess the degree of colonization of the epithelium by fungal hyphae. With this scale, the absence of colonization was given a score of 0, while maximal colonization, where an excess of 50 hyphae could be seen in each high-power field (magnification, ×400) was assigned a score of 4. The scores given were 1 for the presence of 1 to 5 hyphae, 2 for 6 to 15 hyphae, and 3 for 16 to 50 hyphae. The specimens were examined by one of us who was blinded as to the source. Three high-power fields per sample were examined for the light microscopy experiments.

Scanning electron microscopy preparation. Following fixation for 24 h, the tissue was rinsed three times in buffer and postfixed in 1% phosphate-buffered osmium tetroxide (pH 7.4) for 1 h. After two buffer rinses, the specimens were dehydrated in ascending concentrations of ethanol, followed by critical point dehydration in a Denton DCP-1 critical point drying apparatus with liquid CO_2. The tissue samples were affixed on aluminum stubs with silver conductive paint and were sputter coated with gold-palladium by using a Hummer VI sputter-coating apparatus (Anatech Electronics, NJ). Specimens were viewed with a Zeiss (Oberkochen, Germany) 910 electron microscope operated at 20 kV (2).

Statistical analysis. Statistical analysis of the quantitation of *C. albicans* cells in oral tissue was performed by using the Mann-Whitney U test. Data on percentage areas of clinical lesions and percentage of normal papillae were normalized by an arcsine–square root transformation and analyzed with Student's *t* test (differences between stressed and nonstressed groups) or a one-way analysis of variance (ANOVA) followed by Bonferroni's *t* test for post hoc analysis (differences among control, placebo, and fluoxetine groups in the stressed and nonstressed rats). The Kruskal-Wallis test for multiple comparisons was used to determine the degree of colonization of the epithelium by fungal hyphae (35). Differences were considered significant at a *P* value of <0.05.

RESULTS

Candida 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; Fig. 1) were increased in stressed rats compared to nonstressed animals (significant differences, $P < 0.05$). A decrease in the total number of papillae and an increase in the percentage of abnormal (atrophic and hypertrophic) papillae (Table 3) were observed in stressed animals (significant differences, $P < 0.05$). On the semiquantitative

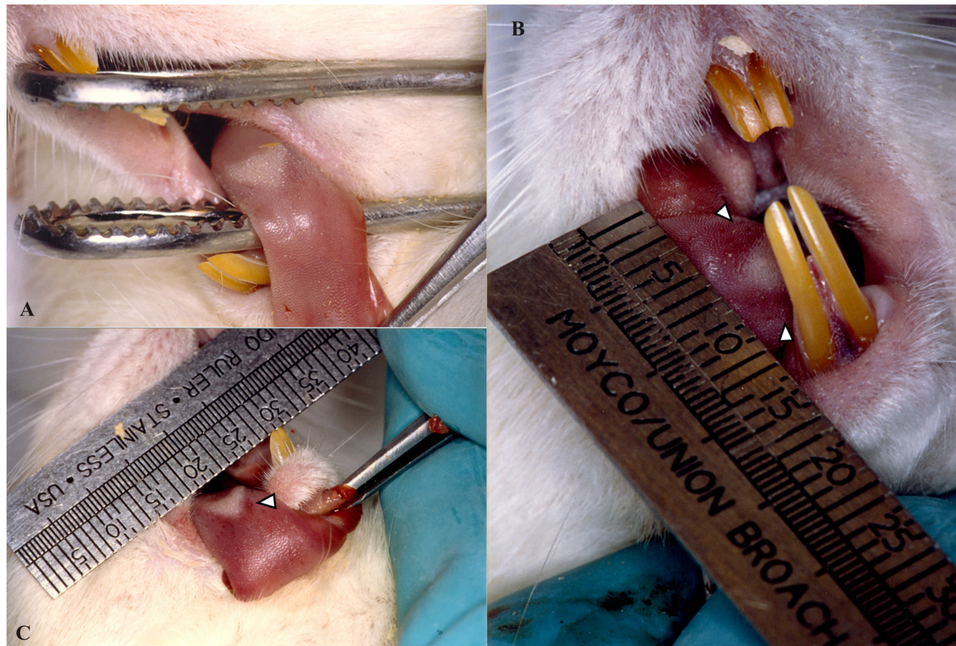

FIG. 1. Clinical lesions in rats 15 days after oral inoculation. Rats were sacrificed and the dorsal tongues of control rats (A), stressed rats injected with placebo (B), and stressed rats injected with fluoxetine (C) were photographed *in situ* at 10× magnification. Note the minor extension of the lesion in stressed rats injected with fluoxetine ccmpared to stressed rats injected with placebo. Arrowheads indicate areas of papilla loss.

scale of colonization of the epithelium by fungal hyphae, stressed rats (Fig. 2) scored higher than untreated controls (significant differences, $P < 0.05$). Neither placebo nor fluoxetine significantly affected those parameters in nonstressed rats ($P > 0.05$), with the only exception that the placebo increased the degree of colonization of the epithelium in nonstressed animals. By contrast, treatment with fluoxetine significantly ($P < 0.05$) reversed the adverse effects of stress by all parameters assayed.

Clinically evident lesions and inflammatory changes of the underlying connective tissue were observed 15 days after *C. 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 (Fig. 3). 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

TABLE 3. Frequencies of normal papillae

Test group	% with normal papillae[a]		
	Controls	Placebo	Fluoxetine
Nonstressed	83.68 ± 3.21	83.4 ± 3.8	87.83 ± 3.12
Stressed	66.2 ± 2.15	64.88 ± 3.21	74.6 ± 4.81

[a] Percentage of normal papillae in the tongues of rats. The results are means ± standard deviations for four animals. Values were analyzed with an arcsin (square root) transformation, ANOVA, and Student's *t* test. Differences between nonstressed and stressed rats with no treatment were significant ($P < 0.05$; $t = 8.193$). Differences between nonstressed and stressed rats injected with placebo were significant ($P < 0.05$; $t = 7.169$). Differences between stressed rats with no treatment and those injected with fluoxetine and between stressed rats injected with placebo or fluoxetine were significant ($P < 0.05$). Differences between nonstressed rats with no treatment and those injected with placebo and between nonstressed rats with no treatment or injected with fluoxetine were not significant ($P > 0.05$).

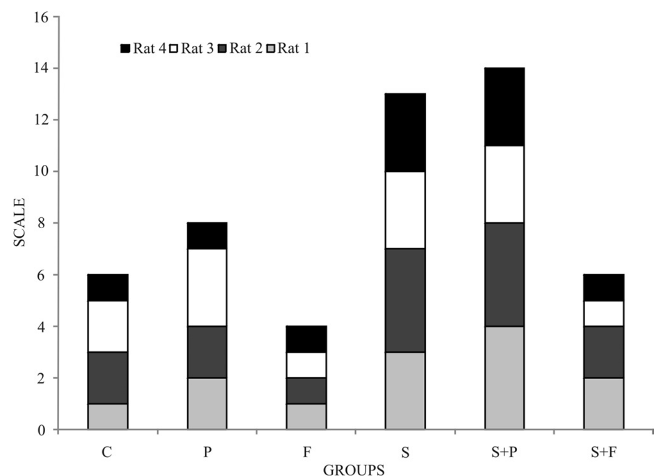

FIG. 2. Semiquantitative scale to assess degree of colonization of epithelium by fungal hyphae. In this scale, the absence of colonization was given a score of 0, while maximal colonization, in which an excess of 50 hyphae could be seen in each high-power field (x400), was assigned a score of 4. The 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. The Kruskal-Wallis test for multiple comparisons was used. Abbreviations: control rats (C), nonstressed rats injected with placebo (P), nonstressed rats injected with fluoxetine (F), stressed rats with no treatment (S), stressed rats injected with placebo (S+P), stressed rats injected with fluoxetine (S+F).

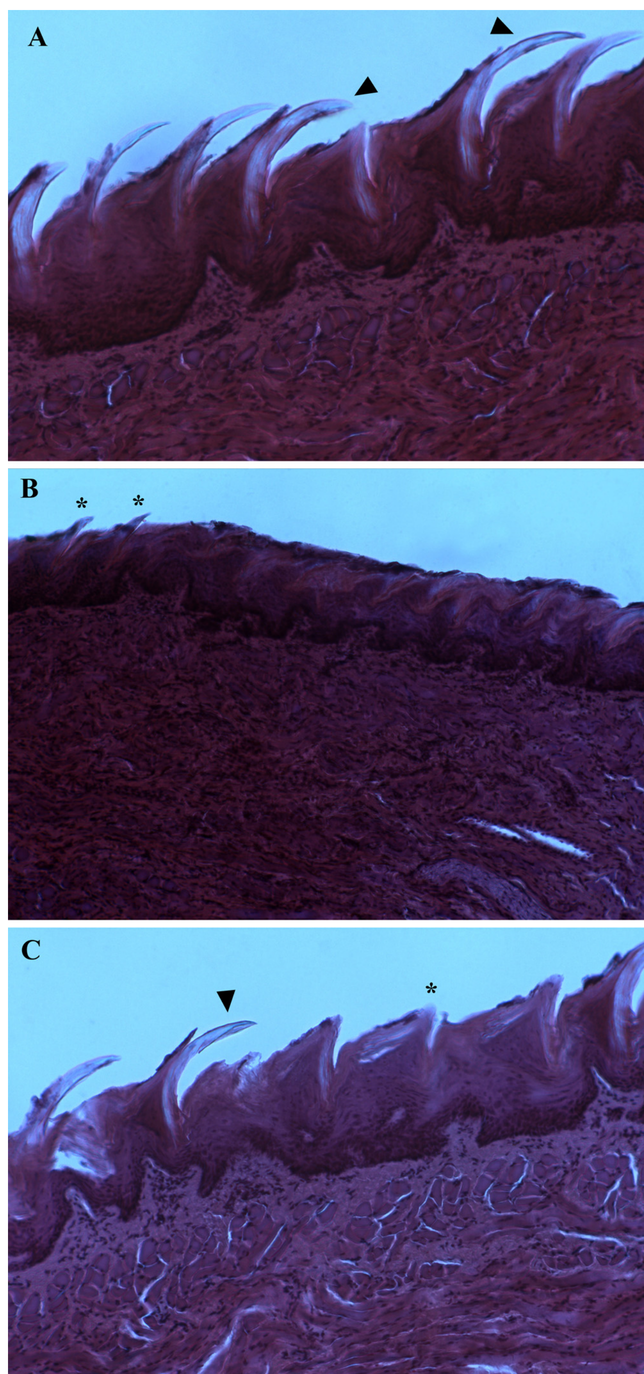

FIG. 3. Qualitative histopathology of rat tongue sections stained with hematoxylin and eosin. (A) Cross-section of representative excised rat tongues from control animals showing the presence of sharp-pointed papillae (arrowheads). (B) Tongue section from stressed rats injected with placebo, showing an almost complete atrophy of the dorsal tongue papillae. Only two papillae (asterisks) have preserved their normal morphology. (C) Tongue cross-section from stressed rats injected with fluoxetine; sharp-pointed (arrowhead) and blunted (asterisk) papillae can be observed. Tongue samples for both groups were taken 15 days after *C. albicans* inoculation.

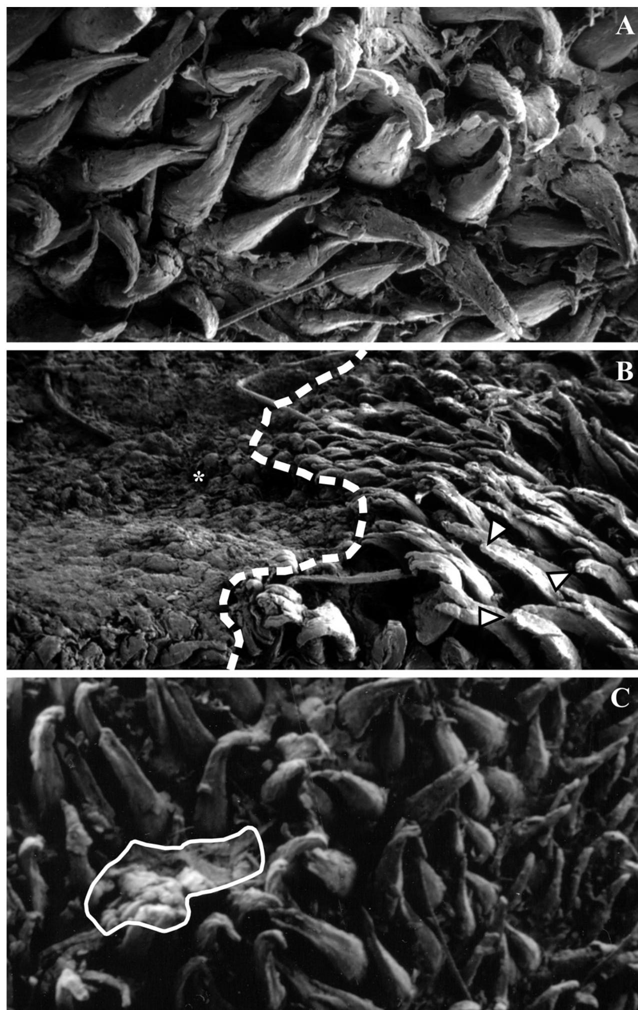

FIG. 4. Scanning electron microscopy images of the dorsal tongues of rats from control (A), stress plus placebo (B), and stress plus fluoxetine (C) treatment groups. (B) The dotted line delineates the boundary between an area of papilla loss (*) and another one with blunted papillae (arrowheads). (C) Note the persistence of a small atrophic area (solid line) surrounded by blunted papillae.

cell infiltrate. Those papillae which supported *Candida* growth appeared shorter and blunter than the surrounding uninfected papillae (Fig. 3).

Scanning electron microscopy (Fig. 4) 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 compared to nonstressed animals. This adverse effect of stress was also reduced by the administration of fluoxetine.

DISCUSSION

Our results show that stress exacerbates *C. albicans* infection of the tongues of rats. Significant increases in *Candida* counts, the percent area of clinical lesions, the percentage of abnormal papillae, and the colonization of the epithelium by fungal hyphae were found in stressed rats compared with those in nonstressed animals. Treatment with fluoxetine partially reversed

those adverse effects of stress on the development of oral candidiasis. Fluoxetine was found to reverse many of the effects of stress on *C. albicans* infection of the tongues of rats, including *Candida* counts, the percent area of clinical lesions, the percentage of abnormal papillae, and the colonization of the epithelium by fungal hyphae.

Stress is thought to increase susceptibility to infections (20, 41, 43), as it has been suggested that stressors, in many cases, are the actual cause of mycosis and the main responsible factor for the recurrence of candidiasis. This hypothesis is supported by studies which generally document the influence of stress on the human immune system (40) and by immunological findings that people with acute or chronic fungal infections exhibit local immune weakness (30) which predisposes them to more frequent relapses (31). Furthermore, clinical and experimental observations indicate that the opportunistic proclivities of *Candida albicans* vary considerably, depending on the nature of the immunological defect of the patient. Patients with qualitative or quantitative defects of phagocytes are mainly prone to the invasive form of this mycosis (10, 14). In contrast, defective T-cell-mediated immunity has been specifically associated with thrush and other forms of candidiasis that are limited to mucocutaneous surfaces (13, 14, 19, 23).

Up to now, nobody has provided conclusive evidence about the incidence of oral candidiasis in fluoxetine-treated and untreated patients, because variables of confusion could be obscuring a possible association between candidiasis and selective serotonin reuptake inhibitors. So, reduced saliva secretion as a consequence of antidepressant medication has been widely reported (24), as dry mouth is a common adverse effect of antidepressants, such as selective serotonin reuptake inhibitors (SSRIs) (42). On the other hand, a higher frequency of *Candida* isolation from the oral cavity of patients under treatment with psychotropic drugs (64.7%) in relation to control individuals (33.3%) has been found (27). Nevertheless, these individuals were wearing complete maxillary dentures, and the isolation frequencies of oral yeasts have been reported to be increased by the presence of dentures (5). In conclusion, the question of whether there is an association between the incidence of oral candidiasis and treatment with antidepressant drugs is still, on the basis of existing studies, an open one.

A number of nonantibiotic drugs (12, 36, 37) exert an influence on the physiology and viability of microorganisms. In 1993, antimicrobial activity was described for psychotropic drugs of the phenothiazine and thioxanthene groups (8). Since then, several substances have been examined, and it has been reported that SSRIs influence the *in vitro* viability of microorganisms. In this regard, it has been observed that fluoxetine enhances *in vitro* susceptibility to chloroquine in *Plasmodium falciparum* (11) and sertraline has *in vitro* and/or *in vivo* antifungal activity against various isolates of *Candida* spp. (25) and *Aspergillus* spp. (26). Nevertheless, the present study shows that fluoxetine *in vivo* does not have direct antifungal effects. This finding does not reject the possibility that fluoxetine could exert an indirect antifungal effect, increasing the activity of drugs against *Candida* spp. which express efflux pumps, according to previously described observations to fluoxetine (11) and other SSRIs (33).

The effects of fluoxetine observed under stress conditions could be direct (with a target cell). Nevertheless, in previous studies with fluoxetine, we observed that this drug did not significantly affect either NK or cytotoxic T-lymphocyte responses when administered in cell cultures (34). Therefore, we cannot suspect direct effects of this drug on immune cells. On the contrary, all evidence seems to indicate that fluoxetine could induce neuroendocrine and neurochemical effects that could indirectly affect immune function, as has been thoroughly discussed by our investigation group (34).

In conclusion, our data show that fluoxetine is effective in countering the adverse effects of stress in stressed mice. Nevertheless, the large number of interactions at molecular, cellular, and functional levels between the nervous system and the immune system that characterize the operational compositions and expressions of the neuroimmune network make complex the isolation of the pathways in which stress and fluoxetine may be involved in the regulation of the host defense mechanisms against infection and of the immune response to stress. Moreover, biological significance and health relatedness of these immunological effects should be assessed.

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