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Effects of Alprazolam on Influenza Virus Infection in Stressed Mice

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FREIRE-GARABAL, M., J. L. BALBOA, J. C. FERNANDEZ-RIAL, M. J. NÚÑEZ AND A. BELMONTE. *Effects of alprazolam on influenza virus infection in stressed mice.* PHARMACOL BIOCHEM BEHAV 46(1) 167–172, 1993. – The review of the literature shows that stress can adversely affect influenza A virus infection. In this report, we study the effects of chronic alprazolam (1 mg/kg/day), a central benzodiazepine agonist anxiolytic, on the influenza A (PR-8/34) virus specific immune injury in mice exposed to a chronic auditory stressor. Treatment with alprazolam resulted in a significant reduction of stress-induced increase of virus titters and pulmonary vascular permeability. A correlation with the lethality of mice was also observed.

Alprazolam Benzodiazepines Influenza A PR8/34 virus Stress Virus infection

THE influence of stress on the incidence and duration of infectious disease is one interesting area of study with particular relevance to health. Previous investigations have described adverse effects of stress on natural and specific immune responses that may predispose the host to more severe acute virus infection (1,30). Changes in murine splenic cytotoxic activities, mediated by natural killer (NK) cells and cytotoxic T-lymphocytes (CTL) have been reported (4,32,40,41,43,49, 53,56). In this regard, stress also interferes with the activity of phagocytosis and T-dependent antibody responses (21,27).

On the other hand, treatment with benzodiazepines, such as alprazolam, was found to reduce some effects of stress on the immune system of mice, such as T-cell depletion and the inhibition of the blastogenic and cytotoxic activities of spleen cells (23,24). Benzodiazepines also promoted the activity of phagocitosis (27), and the resistance to bacteria (25) and cancer (26) in stressed mice.

Nevertheless, there are no data on the effects of benzodiazepines on the response to virus infection in stressed mice. To further elucidate this latter interaction, in the present paper we study the effect of alprazolam on the pathogenicity of the mouse-adapted strain of influenza virus PR-8/34 in mice exposed to a chronic auditory stressor.

METHOD

Mice

Male 3-week-old, pathogen-free, CD-1 mice (Interfauna Ibérica S.A., Barcelona, Spain) were used. They were housed, 7 days before experiments, four per cage in an aseptic and sound-proof chamber kept between 21 and 22°C and maintained on an alternating 12L : 12D cycle. Sterilized food (Panlab Diet A.03) and water were given ad lib.

Procedure

Mice were randomly divided into three groups: group A, unstimulated controls injected with placebo; group B, stressed mice injected with placebo; group C, stressed mice injected with alprazolam.

Induction of Stress

The mice were subjected to a broad band noise at about 100 dB daily for 5 s every minute during 1- or 3-h periods around midnight, at the height of the diurnal activity cycle (40). Unstimulated controls were exposed only to the normal activity of the animal room.

Virus

The mouse-adapted strain of influenza virus PR-8/34 was grown in the allantoic fluid of embryonated eggs after serial passage in mice to increase virulence. Influenza virus infectivity was assayed according to the procedures described by Lennett and Schmidt (39). Log dilutions of lung homogenates were added in a volume of 0.1 ml to the allantoic sac of 10-day-old embryonated chicken eggs. The eggs were maintained at 35°C. After 48 h, the allantoic fluid was harvested and tested at each dilution for the hemagglutination activity. The eggs were scored positive for infectivity at a given virus

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dilution when the allantoic fluid demonstrated positive hemagglutination of chicken erythrocytes (45). The microtiter procedure was used to determine the hemagglutination of influenza virus. The dose required to infect 50% of the embryonated eggs (EID_{50}) was determined by the method of Reed and Muench (46). The virus was inoculated intranasally 24 h after the beginning of drug administration.

Lethality Assay

Mice were inoculated intranasally with 1 HAU/mouse (20 mice/group) and 5 HAU/mouse (20 mice/group) PR8/34 virus. Survival percentages were recorded every second day during 16 days (22).

Virus Titters

The pathogenesis of influenza virus infection in mice was determined by quantization of virus titters (45). Mice were inoculated intranasally with 0.1 LD_{50} of influenza A virus. After 12 and 24 h, and 3, 6, and 9 days of infection, six mice from each group were sacrificed by cervical dislocation and immediately necropsied. Their lungs were removed under aseptic conditions, minced with scissors, weighed, and stored at −70°C. A 20% (w/v) lung suspension was prepared by homogenizing lung tissue in Hank's balanced salt solution. This suspension was clarified by centrifugation at 500 × *g* for 10 min at 4°C. The infectious virus was assayed in embryonated eggs as previously described (45).

Calculation of Alveolar Capillary Leak

Permeability index (PI), which indicates increased pulmonary vascular permeability, was calculated to determine tissue injury (33,45). After 3, 6, 9, 12, and 15 days of inoculation with 0.1 LD_{50} influenza A virus, [^{125}I]-labeled bovine serum albumin (100 μl = 140,000 cpm) was injected into the tail vein of six mice from each experimental group. After 2 h, the mice were anesthetized with ketamine (150 mg/kg), 100 μl of caval blood was withdrawn, and the heart and lungs removed en bloc (45). The pulmonary vasculature was perfused with 2 ml saline via the right ventricle to remove the blood-associated radioactivity in the pulmonary system. Lung tissue and blood were counted in a gamma counter (LKB Wallac 1275 Gamma Counter). The ratio of radioactivity present in the lungs to that present in the animal's blood was expressed as the PI.

Drug Treatments

Alprazolam (Upjohn, USA) was intraperitoneally (IP) injected at a dosage of 1 mg/kg, in a volume of 1 ml/kg of 1% water solution of carboxymethylcellulose as vehicle. Control-unstressed and control-stressed mice were IP injected with 1 ml/kg of diluent as placebo. Drugs were administered daily at 0930 h during all periods of stress application.

Statistical Analysis

Mortality ratios and mean times to death were analyzed with Fisher's exact test and the Mann-Whitney *U*-test, respectively. Analysis of mean differences in virus titters and PI was performed according to the Student's *t*-test. Significance was achieved at $p < 0.05$.

RESULTS

The effects of stress and alprazolam on the survival of PR8/34-infected mice are shown in Fig. 1. Stress significantly decreased the resistance to PR8/34 infection. This effect was very evident at 5 HAU/mouse. Calculation of mean time to death showed that significantly earlier times of death occurred among mice of group B (4.9 ± 0.8 days after infection) in comparison with mice of group A (11.4 ± 0.2 days after infection). Treatment with alprazolam partially reversed the adverse effect of stress (mean time to death in group C: 6.7 ± 0.5 days after infection).

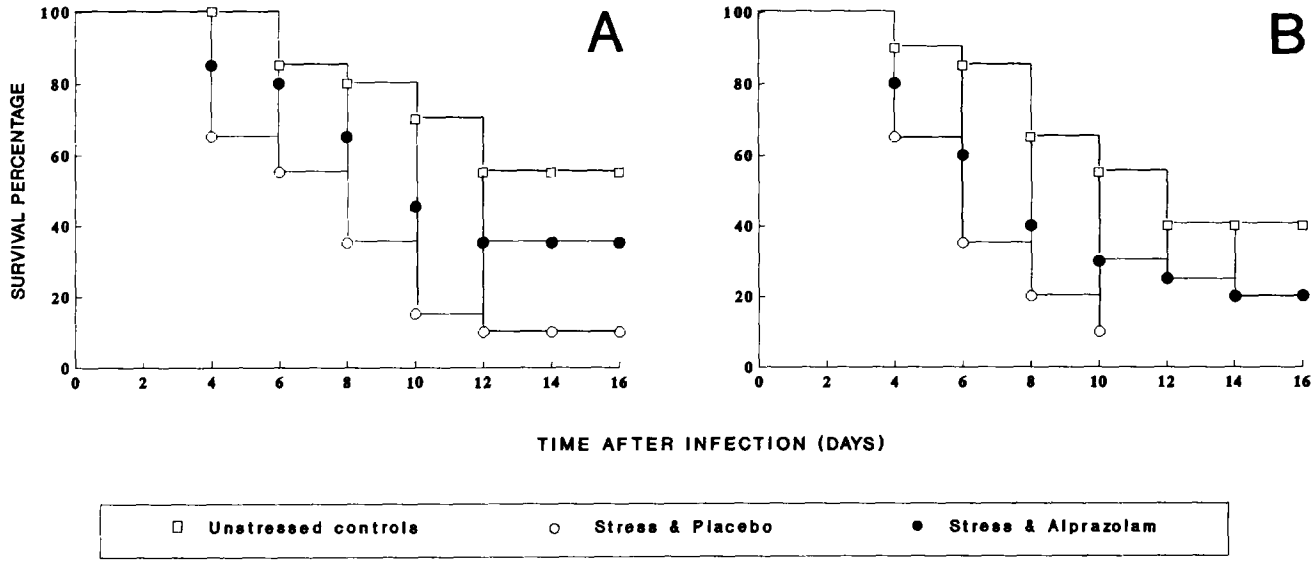

FIG. 1. Effect of stress and alprazolam on the survival of PR8/34-infected BALB/c mice. Twenty-four hours after the beginning of experiments, mice were inoculated intranasally with PR8 virus [(A) 1 HAU/mouse; (B) 5 HAU/mouse]. Survival curves represent the values of two experiments. $p < 0.05$ for the mortality ratios.

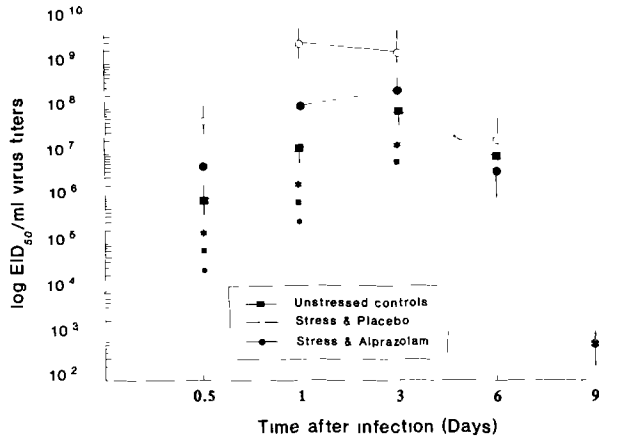

FIG. 2. Virus titers in lung tissue of mice infected with 0.1 LD_{50} influenza A virus and sampled at given times after inoculation. Six animals from each group were sampled at each time. *Differences between unstressed controls and stressed mice injected with placebo significant at $p < 0.05$. ■Differences between stressed mice injected with placebo and stressed mice injected with alprazolam significant at $p < 0.05$. ●Differences between unstressed controls and stressed mice injected with alprazolam significant at $p < 0.05$.

Virus titers in the lungs of mice sacrificed 12 h, 24 h, and 3 days after infection were greater in group B in comparison with group A. Group C values were significantly smaller compared with those of group B in the same assays (Fig. 2). Differences between groups C and A were only significant on the 12-h and 24-h assays.

Figure 3 shows the alveolar capillary leakage during the influenza A pulmonary infection in mice infected with a sublethal concentration (0.1 LD_{50}) of virus. Permeability index was significantly greater in group B mice in comparison with group A mice until the 12th day after inoculation. Treatment with alprazolam (group C) resulted in significant reduction of the PI in comparison with group B until the 9th day of experiments. Values in group C were significantly higher than those of group A on the 9th day after infection.

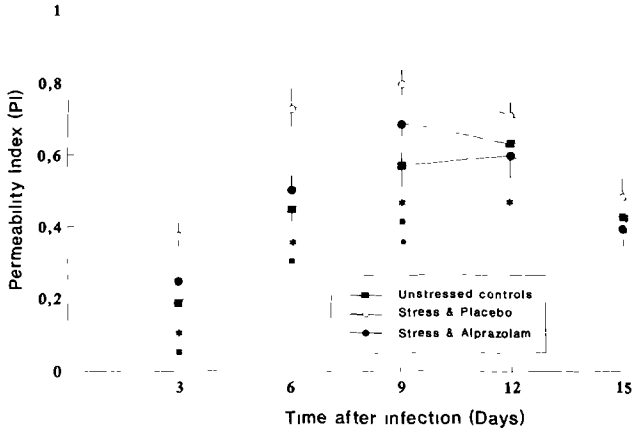

FIG. 3. PI for mice infected with 0.1 LD_{50} influenza A virus and sampled at given times after inoculation. Six animals from each group were sampled at each time. *Differences between unstressed controls and stressed mice injected with placebo significant at $p < 0.05$. ■Differences between stressed mice injected with placebo and stressed mice injected with alprazolam significant at $p < 0.05$. ●Differences between unstressed controls and stressed mice injected with alprazolam significant at $p < 0.05$.

DISCUSSION

The mouse-adapted strain of influenza virus PR-8/34 is highly lethal in mice and affects several immune functions, such as NK activity, delayed hypersensitivity response, and IL-2 production (21).

Our results show that stress exacerbates influenza virus infection. Calculation of mean time to death indicated that significantly earlier times of death occurred among mice submitted to stress compared with unstressed controls. By contrast, the onset of death following inoculation was delayed in mice injected with alprazolam.

Pathologic findings in this report show that influenza A virus titters in the lungs of mice were greater for stressed mice injected with placebo in comparison with unstressed controls until the 3rd day. Furthermore, alveolar capillary leakage was greater in this group until the 12th day. Treatment with alprazolam in stressed mice resulted in lower virus titters and lower permeability index in comparison with placebo in most of the intervals cited above.

The results presented here are in good agreement with previous studies on the adverse effects of stress on virus infection. Broadbent et al. (15) reported that virus shedding in subjects experimentally exposed to rhinoviruses and influenza viruses was related to their personality status as either introverts or extroverts. Individuals who reported greater distress on a self-report inventory had more evidence of nasal secretion after infection. Clover et al. (19) found that infection with influenza B virus was associated with family cohesion and adaptability, suggesting that family dysfunction may be related to altered immune response. Friedmann et al. (29) found that mood was negatively associated with cold sore recurrence in 149 nurses. Other authors (13) found that infectious bovine rhinotracheitis viral growth in bovine kidney cell cultures was enhanced fourfold in cultures with serum from stressed calves. A series of studies have also reported the relevance of stress on other types of virus infection, such as HSV (31,34,35,50,55).

Research has proved the role of thymus-dependent immune response in influenza A virus-infected mice. The development of T-cells with cytotoxic activity specific for influenza virus-infected cells appears to be the critical factor in limiting the viral infection to the respiratory tract and in recovery (21). Transfer of a population of lymphocytes containing cytotoxic T-cell activity from BALB/c mice infected with influenza A virus 8 days previously results in an earlier and greater reduction in pulmonary virus titters in T-cell-deficient recipient mice (59). Compared to immunocompetent controls, nude mice also showed a higher mortality following influenza A inoculation, and those mice that survived tended to have persistent viral presence in their lungs (59). By contrast, the onset of death following inoculation was somewhat delayed in the nude mice group.

By contrast, experiments with cyclosporine A-treated mice are not fully in line with our findings (14,48). This drug acts early during the process of T-cell activation by blocking the release of lymphokines, thereby preventing the formation of effector T-cells. Cyclosporine A, when administered to BALB/c mice with a moderate-size inoculation of influenza A virus, substantially alters the immune response to the infec-

tion. Nevertheless, although infected mice treated for 21 days with cyclosporine had higher virus titters in their lungs than infected controls not exposed to the immunosuppressive agent, cyclosporine-induced suppressive effects of certain populations of T-cells seemed to be beneficial in reducing the pathogenesis of influenza A virus pulmonary infection, as the histopathology seen in the lungs was less severe.

The adverse effects of different schedules of stress on the number and functional capacities of T-cells in mice have been widely documented. Changes in the absolute number of lymphocytes, T-lymphocytes, and T-helper and T-suppressor cells of rodents have been reported (23,24,53). Stress also interferes with several immune responses such as murine splenic cytotoxic activities, mediated by NK cells and CTL (4,32,40,41, 43,49,53,56). Stress also suppresses other aspects of cell-mediated immunosurveillance, such as phagocytic activity and T-dependent antibody responses (21,27). Therefore, changes in the pathogenicity of influenza A virus should be attributed to the stress-associated immunosuppression (21).

The mechanism by which stress inhibits the cellular immune response seems to be complex. A molecular basis for bidirectional communication between the immune and neuroendocrine systems has been described (1,5–12,17,47,51,52). Cell to cell communications between the immune and the neuroendocrine systems are 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 (2,16, 58). Moreover, receptor sites are present in lymphoid cells for many hormones and neurotransmitters (8,58). 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 very much like those produced by the hypophysis, such as ACTH, TSH, GH, PRL, gonadotrophin, and β-endorphin (5, 8,57,58).

On the other hand, treatment with alprazolam resulted in a significant reduction of the pathogenicity of influenza A virus in stressed mice. These data are in line with our previous studies showing that alprazolam reversed the suppressive effects of stress on T-cell populations, the blastogenic response of spleen cells, the NK and CTL responses, and the phagocytic activity (23,24,27). Protective effects of benzodiazepines on immune functions were also reported by Fride et al. (28), who found immunoenhancing effects of alprazolam in mice. Okimura and Nagata (43) observed that diazepam promoted the antibody response through stimulating helper T-cell functions in restraint-stressed mice. By contrast, Chang et al. (18) found some suppressive effects of alprazolam on the immune response of mice.

The mechanism of action of benzodiazepines 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 benzodiazepine (BZD) receptors acting by facilitating inhibitory GABA neurotransmission in CNS may regulate the release of neuroendocrine hormones involved in the immune response to stress. For example, the rise in plasma corticosterone, via ACTH secretion, which has an easily demonstrable destructive effect on specific cells and tissues that are required for optimum immunological defense (3,38,42,46,47,54), is partially reversed by the administration of benzodiazepines (36,38,44,54). Nevertheless, Fride et al. (28) found that the inmunoenhancing effects of alprazolam in the absence of stress did not appear related to corticosterone levels. In contrast, vehicle injection caused a profound suppression of several immune parameters that appeared to correlate with a concurrent rise in serum corticosterone levels.

In previous studies not reported here, we observed that alprazolam reduced the stimulatory effect of a stress on the secretion of β-endorphin. This latter peptide has also been implicated in the immunosuppressive effects of stress. Other hormones such as PRL and melatonin should be involved.

A second aspect is the existence of a benzodiazepine receptor with high affinity on immune cells that express the so-called peripheral specificity for several benzodiazepines (60). Nevertheless, alprazolam is described in the literature as a strict central-type ligand of the BZD receptor (20,28), whereas alprazolam has potent PAF antagonist properties (37). Recent data published on the action of alprazolam in vitro on T-, B-cell, and macrophage responses may well reflect this type of anti-PAF activity (18).

In conclusion, our data at present show that stress, through known and unknown neuroendocrine pathways, should injure the elements of the immunological apparatus, which may leave the subject vulnerable to the action of viruses. Alprazolam, a benzodiazepine agonist, was found to have beneficial effects against adverse effects of stress. Nevertheless, biological significance and health relatedness of our data should be assessed.

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