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Suppression of Natural Killer Cell Activity by FG 7142, a Benzodiazepine Receptor "Inverse Agonist"¹

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A dose-dependent (5-50 mg/kg) suppression of natural killer (NK) cell activity was observed 2 h after administration of the benzodiazepine receptor "inverse agonist" FG 7142 (*N*-methyl- β -carboline-3-carboxamide), and was still manifest 24 h later. Addition of FG 7142 (1-1000 nM) to the 4 h ⁵¹Cr release assay did not affect NK cell activity. Pretreatment of mice with the benzodiazepine receptor antagonist Ro 15-1788 (10 mg/kg) blocked FG 7142-induced suppression of NK cell activity, but had no effect when administered alone. The suppression of NK cell activity by FG 7142, a compound which produces a syndrome resembling stress or anxiety in both animals and man, provides further evidence that the central nervous system pathways subserved by the benzodiazepine/GABA receptor chloride channel complex ("supramolecular complex") may play a role in the modulation of immune function. © 1989 Academic Press, Inc.

INTRODUCTION

Natural killer (NK) cells, in addition to being important in the defense against viral infections, play a key role in the body's surveillance against neoplastic diseases (Herberman & Ortaldo, 1981; Welsh, 1987). Both basic and clinical studies suggest that some forms of anxiety, environmental stress, and major depression are associated with suppression of NK cell activity (Shavit, Lewis, Terman, Gale, & Leibeskind, 1984; Locke et al., 1984; Kiecolt-Glaser et al., 1984; Irwin, Smith, & Gillin, 1987). We recently reported that the administration of the benzodiazepine receptor (BzR) "inverse agonist" (whose actions are best described as pharmacologically opposite to benzodiazepines, for review see Skolnick and Paul, 1983) FG 7142 (*N*-methyl- β -carboline-3-carboxamide), produced a profound suppression of T-cell functions (Arora, Hanna, Paul, & Skolnick, 1987). Since β -carbolines like FG 7142 have been demonstrated to elicit a BzR-mediated behavioral, somatic, and endocrine syndrome resembling stress or anxiety in both animals and man (Ninan et al., 1982; Dorow, Horowski, Paschelke, Amin, & Braestrup, 1983; Insel et al., 1984; File & Pellow, 1985), our findings (Arora et al., 1987) suggested that the benzodiazepine receptors in the central nervous system

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(CNS) and the pathways subserved by these receptors may be important in the neural modulation of immunity. In the present study, we investigated whether FG 7142 administration would also influence NK cell activity.

We found that FG 7142 produced a dose-dependent suppression of NK cell activity, and that this suppression was reversed by pretreatment of mice with a BzR antagonist, Ro 15-1788. Moreover, direct addition of FG 7142 (1–1000 nM) to ^{51}Cr release assays did not affect NK cytotoxicity. These findings support the hypothesis that the CNS pathways subserved by the benzodiazepine receptors may be important in the neural modulation of immunity.

MATERIALS AND METHODS

Animals

Male B10.BR/SgSnJ mice were obtained from the Jackson Laboratories (Bar Harbor, ME). Mice were housed in plastic cages (≤ 5 animals/ $6 \times 10 \times 5$ -in. cage) on a 12-h light cycle (lights on at 0700) with free access to food and water. All animals used in these experiments were approximately 8 weeks old and had been acclimated to our vivarium for 14 days prior to use.

Drugs

FG 7142 was purchased from Research Biochemicals (Wayland, MA). Ro 15-1788 was donated by Hoffmann-La Roche (Nutley, NJ). Emulphor was the gift of GAF Corp. (Wayne, NJ). Drugs were suspended in 20% diluted Emulphor/80% saline (Arora et al., 1987) just prior to use and injected intraperitoneally in a volume of 0.15 ml (FG 7142) or 0.1 ml (Ro 15-1788).

Preparation of Spleen Cells

Mice were killed by cervical dislocation between 0800 and 1000 h. Spleens were immediately removed and placed in Hanks' balanced salt solution (HBSS) (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories, Logan, UT) and 10 mM Hepes buffer (GIBCO). Following the mechanical dissociation of individual spleens into a single cell suspension, ammonium chloride lysing solution (ACK lysing buffer, NIH Media Unit, Bethesda, MD) was added for 2.5 min to remove the erythrocytes. Subsequently, the cells were washed twice in HBSS + 10% FBS and counted by trypan blue exclusion.

Measurement of NK Cytotoxic Activity

Spleen cells were tested for NK cytotoxicity using a ^{51}Cr release assay (Arora & Shearer, 1981). Briefly, varying dilutions of spleen cells in 100- μl aliquots were added in quadruplicate to microtiter U-bottom wells (COSTAR, Cambridge, MA). Target cells used were NK-sensitive YAC-1 tumor cell line labeled with 200 μCi of ^{51}Cr ($\text{Na}_2^{51}\text{CrO}_4$; DuPont-NEN, Boston, MA). After three washings in medium containing 10% FBS, target cells were counted and added (100- μl aliquots) in quadruplicate to microtiter wells containing effector spleen cells. The plates were centrifuged for 3 min at 400 rpm and incubated at 37°C for 4 h in a 95% air, 5% CO_2

atmosphere. After the incubation, the plates were centrifuged for 5 min at 800 rpm, and the supernatant was collected with the Titertek Supernatant Collection System (Skatron Inc., Sterling, VA) and counted in a Beckman Auto Gamma scintillation spectrometer. The percentage of lysis was determined as described (Arora & Shearer, 1981) using the equation

$$\% \text{ Lysis} = \frac{\text{Exp. CPM} - \text{Spont. CPM}}{\text{Max. CPM} - \text{Spont. CPM}} \times 100,$$

where Exp. CPM was defined as the counts released into the medium by target cells when assayed with effector spleen cells, Max. CPM was defined as the counts released by target cells in the presence of 2.5% Triton X-100 (no effectors), and Spont. CPM was defined as the counts released by target cells in the presence of medium alone. Spontaneous release ranged from 10 to 15% of the maximum ^{51}Cr release.

Data were analyzed by analysis of variance (ANOVA) followed by a Fisher's protected least-significant difference test where indicated.

RESULTS

Effects of FG 7142 on NK Cell Activity

A dose-dependent suppression of NK cell activity was observed 2 h after administration of FG 7142 (Fig. 1). At the E:T ratio of 50:1 illustrated, this suppres-

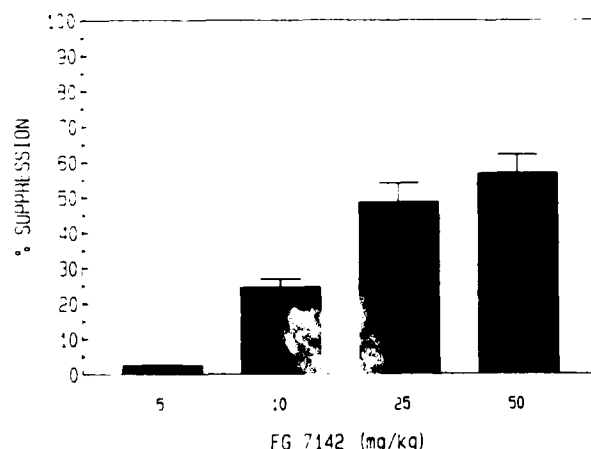


FIG. 1. Inhibition of NK cell activity by FG 7142: dose-response relationship. Male B10.BR mice were injected with FG 7142 (5–50 mg/kg) or an equal volume of vehicle. Spleens were removed 2 h later and NK cell activity was measured as described under Materials and Methods. Values represent the $\bar{X} \pm \text{SEM}$ ($n = 10$) at an effector:target cell (E:T) ratio of 50:1. Data is presented as the percentage of suppression of NK cell activity since it represents two independent experiments, each compared with their respective vehicle-injected controls. Similar dose-response relationships were observed at the other E:T ratios tested (100:1 and 25:1). Analysis of variance followed by a Fisher's protected least-significant difference test indicated that the 25 and 50 mg/kg groups were significantly different from the vehicle-treated group ($p < .01$).

sion ranged from 2.5 to 56% of control values (ANOVA: $F(4,25) = 6.36$, $p < .005$). Post hoc analysis revealed that the treatment groups receiving 25 and 50 mg/kg of FG 7142 were significantly different from vehicle-treated mice ($p < .01$). A similar dose-dependent suppression of NK cell activity was also observed at other E:T ratios (100:1 and 25:1) examined (data not shown). As seen in two independent experiments (Fig. 2), 24 h after administration of FG 7142 (25 mg/kg), a statistically significant suppression of NK cell activity was still evident (42.4% in Exp. 1 and 25.3% in Exp. 2) (Fig. 2). Direct addition of FG 7142 (1–1000 nM) to the 4-h ^{51}Cr release assay did not significantly affect NK cell activity (Fig. 3).

Antagonism of FG 7142-Induced Suppression of NK Cell Activity by Ro 15-1788

Pretreatment of mice with Ro 15-1788 (10 mg/kg) 15 min prior to administration of FG 7142 (25 mg/kg) resulted in a significant reduction in the FG 7142-induced suppression of NK cell activity (Fig. 4). Thus, in this series of experiments FG 7142 (25 mg/kg) suppressed NK cell activity by 35.6% ($p < .05$, compared with vehicle-treated animals) and this suppression was reduced to 16.6% ($p > .05$, compared with vehicle-treated animals) in mice pretreated with Ro 15-1788 (Fig. 4). Ro 15-1788 did not reduce NK cell activity when administered alone.

DISCUSSION

These experiments demonstrate that the BzR inverse agonist FG 7142 produces a dose-dependent suppression of NK cell activity against the lymphoma tumor target, YAC-1. This suppression was manifest 2 h after FG 7142 administration, and was still evident 24 h later. The doses of FG 7142 needed to suppress NK cell activity are consistent with those that produce both behavioral and endocrine

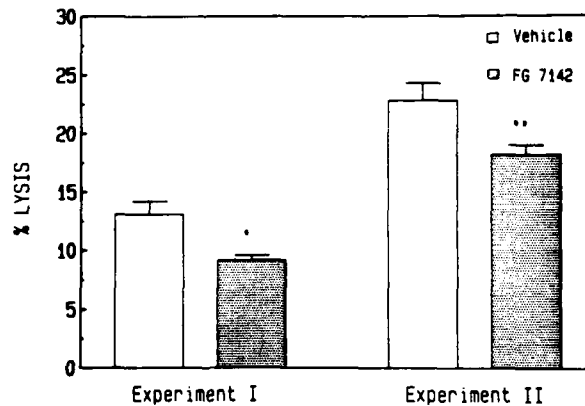


FIG. 2. Inhibition of NK cell activity by FG 7142. Splenic NK cell activity was measured in male B10.BR mice 24 h after an injection of FG 7142 (25 mg/kg) or an equal volume of vehicle. Values represent $\bar{X} \pm \text{SEM}$ ($n = 12$) of data and are expressed as the percentages of lysis of YAC-1 target cells at an E:T ratio of 50:1. A statistically significant suppression of NK cell activity was found ($t(22) = 3.66$, $p < .005$; indicated by *) in both experiments.

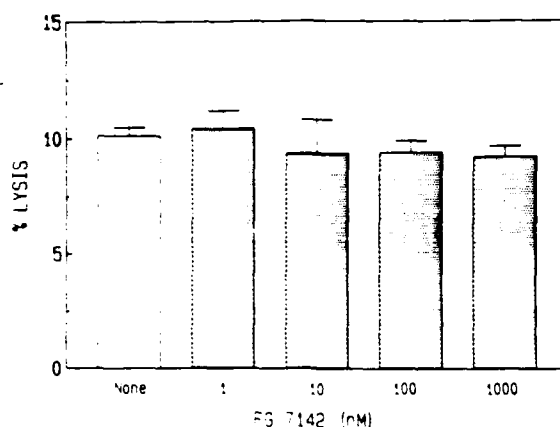


FIG. 3. Effect of *in vitro* addition of FG 7142 on NK cell activity. FG 7142 (1–1000 nM) was added to 4 h ^{51}Cr release assay (for NK cell activity). Data is presented as percentage of lysis ($\bar{X} \pm \text{SEM}$ of quadruplicate samples) of YAC-1 target cells at an E:T ratio of 50:1.

changes in rodents reminiscent of stress or anxiety (File & Pellow, 1985; Stephens & Kehr, 1985) and those that suppressed T-cell functions (Arora et al., 1987).

Several lines of evidence suggest that the suppression of NK cell activity by FG 7142 is mediated via occupation of BzR in the central nervous system. Direct addition of FG 7142 (1–1000 nM) to the ^{51}Cr -release assay during a 4-h incubation period had no effect on NK cell activity (Fig. 3). Furthermore, neither Ro 15-1788 nor β -carbolines such as FG 7142 bind with high affinities to "peripheral" BzR (Marangos, Patel, Boulenger, & Clark-Rosenberg, 1982; Schoemaker, Boles,

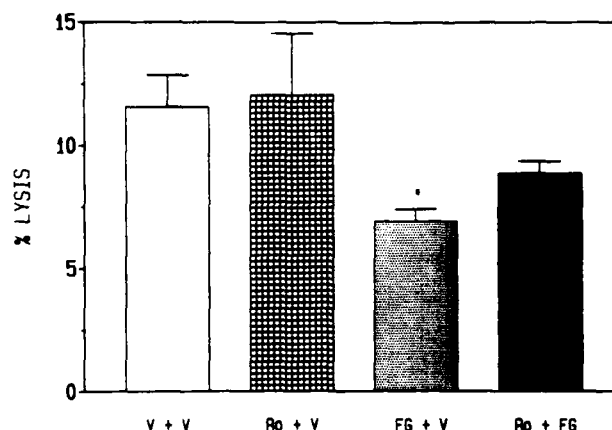


FIG. 4. Ro 15-1788 blocks FG 7142-induced suppression of NK cell activity. Male B10.BR mice were pretreated with either Ro 15-1788 (10 mg/kg) or an equal volume of vehicle. Fifteen minutes later animals were given either FG 7142 (25 mg/kg) or vehicle. Spleens were removed 2 h later and NK cell activity was determined as described under Materials and Methods. Values are expressed as percentages of lysis ($\bar{X} \pm \text{SEM}$ of five to eight mice) of YAC-1 target cells at an E:T ratio of 50:1. Symbols: V, vehicle; Ro, Ro 15-1788; FG, FG 7142.

Horst, & Yamamura, 1983) that are present on cells of the immune system (Zavala, Haumont, & Lenfant, 1985; Ruff, Pert, Weber, Wahl, & Paul, 1985; Moinjeon, Bidart, Alberici, & Bohuon, 1983; Zavala & Lenfant, 1987). Finally, the antagonism of FG 7142-induced suppression of NK cell activity by Ro 15-1788 (a specific, high-affinity BzR antagonist) is consistent with the ability of this compound to block the effects of both BzR agonists (i.e., substances with benzodiazepine-like qualities) and inverse agonists (Skolnick & Paul, 1983).

In our previous study examining the effects of BzR inverse agonists on T-cell functions, Ro 15-1788 administered 30 min prior to FG 7142 elicited a statistically significant but incomplete blockade of the inhibition of mitogen-stimulated T-cell proliferation produced by FG 7142 (Arora et al., 1987). In an attempt to circumvent the rapid *in vivo* degradation of Ro 15-1788 in rodents ($t_{1/2}$ 15 min) (Lister, Greenblatt, Abernathy, & File, 1984) a shorter interval between drugs (15 versus 30 min) was chosen in the present study. While Ro 15-1788 reduced the FG 7142-induced suppression of NK cell activity to values that were not statistically significantly different from vehicle-treated mice, NK cell activity (16.6%) remained slightly suppressed (Fig. 4). Experiments using metabolically stable BzR antagonists and/or multiple injections of Ro 15-1788 may be required to determine whether the small residual suppression of both NK and T-cell functions is attributable to pharmacokinetic or pharmacodynamic factors.

While the mechanism(s) by which FG 7142 suppressed NK cell activity is unclear, the observed immune suppression may be due to changes in the circulating levels of cytokines (catecholamines, β -endorphin, and glucocorticoids) and/or lymphokines (α -interferon and interleukin-2) all of which have been shown to affect NK cell activity (Parillo & Fauci, 1978; Hellstrand, Hermanson, & Strannegard, 1985; Morely, Kay, Solomon, & Plotnikoff, 1987; Irwin, Daniels, Risch, Bloom, & Weiner, 1988). Other possible mechanisms of observed NK suppression may be due to FG 7142-induced changes in recirculation patterns of splenic NK cells (Williams et al., 1981) and/or changes in regulatory cell function (Herberman & Santoni, 1984). These findings suggest that BzR inverse agonists are useful tools to study neural-immune interactions and support the hypothesis (Arora et al., 1987) that the pathways subserved by the "supramolecular complex" may play an important role in the neural modulation of immunity.

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