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Suppression of the immune response by benzodiazepine receptor inverse agonists

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Summary

24 h after administration of a single dose of the benzodiazepine receptor inverse agonists *N*′-methyl-β-carboline-3-carboxamide (FG 7142) and 3-carbomethoxy-4-ethyl-6,7-dimethoxy-β-carboline (DMCM), a profound suppression of the immune response was observed in rodents. This immunosuppression was manifest as a decrease in phytohemagglutinin (PHA) and concanavalin-A (Con-A) stimulated T cell proliferation in rats and mice administered FG 7142 and a decrease in allogeneic cytotoxic T lymphocyte activity in mice administered either FG 7142 or DMCM. The effects of FG 7142 were antagonized by the prior administration of Ro 15-1788, a benzodiazepine receptor antagonist. These findings demonstrate that the neural pathways subserved by benzodiazepine receptors can modulate immune function, and suggest that these receptors may be involved in the stress-induced modulation of immune function.

Abbreviations: FG 7142, *N*′-methyl-β-carboline-3-carboxamide; DMCM, 3-carbomethoxy-4-ethyl-6,7-dimethoxy-β-carboline; PHA, phytohemagglutinin; Con-A, concanavalin-A; CNS, central nervous system; HPA, hypothalamic-pituitary-adrenal; HBSS, Hanks' balanced salt solution; FBS, fetal bovine serum; TCM, tissue culture medium; CTL, cytotoxic T lymphocyte.

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Introduction

While the concept of a neural modulation of immunity remains controversial (Angell 1985; Hall 1985) both psychosocial and environmental stressors have been shown to affect the humoral and cellular components of immunity in laboratory animals and man (Ader 1981; Tecoma and Huey 1985). Benzodiazepine receptors in the central nervous system (CNS) mediate the principal pharmacological actions of the benzodiazepines (Mohler and Okada 1977; Squires and Braestrup 1977), and have also been implicated in the physiological control of stress and anxiety (Ninan et al. 1982; Dorow et al. 1983; Insel et al. 1984; Havoundjian et al. 1986).

The CNS pathways which ultimately affect stress-induced alterations of immune function are unknown, but presumably involve integration of both neocortical and limbic inputs (Biziere et al. 1985; Roszman et al. 1985). High densities of benzodiazepine receptors are found in the mammalian telencephalon, particularly in limbic and neocortical areas (Braestrup et al. 1977; Young and Kuhar 1979) and there is evidence which suggests that these sites may be important in the physiological expression of stress and anxiety (Mohler and Okada 1977; Ninan et al. 1982; Dorow et al. 1983; Insel et al. 1984; Havoundjian et al. 1987). For example, administration of benzodiazepine receptor 'inverse agonists' such as FG 7142 elicits a somatic, endocrine, and behavioral syndrome resembling stress or anxiety in rodents and primates, including man (Ninan et al. 1982; Dorow et al. 1983; Insel et al. 1984). Moreover, stress-induced changes in both the number and permeability characteristics of benzodiazepine receptor-coupled chloride channels occur simultaneously with, or may precede activation of the hypothalamic-pituitary-adrenal (HPA) axis (Havoundjian et al. 1987). These observations prompted a study of the potential importance of pathways subserved by benzodiazepine receptors in the neural control of immunity.

Materials and methods

Animals

Adult male Sprague-Dawley rats were obtained from Taconic Farms, Germantown, NY. Male NFR/N mice were obtained from Veterinary Resources Branch, NIH. Male BALB/c mice were purchased from Charles River, Wilmington, MA.

Reagents

FG 7142 (*N*′-methyl-β-carboline-3-carboxamide) and DMCM (3-carbomethoxy-4-ethyl-6,7-dimethoxy-β-carboline) were purchased from Research Biochemicals, Wayland, MA. Emulphor was the gift of GAF Corporation, New York, NY. Emulphor was diluted 1:1 (w/w) with ethanol to make diluted Emulphor. β-Carbolines were dissolved in the vehicle (20% diluted Emulphor/80% saline) just prior to use. Rats were administered either FG 7142 (10 mg/kg) or an equal volume (0.15 ml) of vehicle. Mice received FG 7142 (25 mg/kg), DMCM (3 mg/kg), or an equal volume (0.1 ml) of vehicle. The benzodiazepine receptor antagonist Ro

15-1788 was obtained from Hoffmann-La Roche, Nutley, NJ. Rats were administered with Ro 15-1788 (10 mg/kg) or an equal volume of vehicle 30 min prior to administration of FG 7142 (10 mg/kg) or vehicle. All drugs were administered intraperitoneally. Mitogens concanavalin-A (Con-A) and phytohemagglutinin (PHA) were purchased from Sigma Chemical Co., St. Louis, MO.

Preparation of spleen cells

24 h after injection, the animals were sacrificed and the spleens removed immediately and placed in sterile saline. Single-cell suspensions from individual spleens were prepared as previously described (Arora and Shearer 1981). In brief, spleens were minced, passed through a sterile nylon screen, and washed in Hanks' balanced salt solution (HBSS) (Gibco, Grand Island, NY). The cell pellet was treated with ammonium chloride lysing solution (ACK lysing buffer, NIH Media Unit, Bethesda, MD) for 2 min to remove erythrocytes, washed with HBSS, and counted by trypan blue exclusion. The viable cell count did not differ among treatment groups.

Mitogen-stimulated T cell proliferation

Mitogen- (Con-A and PHA) stimulated T cell proliferation was determined as follows: spleen cells were placed in 96-well flat bottom microtiter plates (Costar, Carbundy, MA) at 2×10^5 cells/well in 200 μl of tissue culture medium (TCM) consisting of RPMI-1640 (Gibco) medium supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, penicillin (100 U/ml), streptomycin (100 μg/ml), and 5% heat-inactivated fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT). Cells were cultured in the presence or absence of mitogens for a total of 72 h (Con-A was added to cultures in a final concentration of 0.5–1 μg/culture and PHA at a final concentration of 5 or 10 μg/culture, and pulsed with 1 μCi of [^{3}H]thymidine (spec. act. 6.7 Ci/mmol, New England Nuclear, Boston, MA) for the final 18–20 h. The cells were harvested with a MASH II Cell Harvester and [^{3}H]thymidine uptake measured in a liquid scintillation counter. Stimulation indices (SI) were defined as the quotient of [^{3}H]thymidine incorporated in the presence and absence of mitogens. Each concentration of mitogen was assayed in quadruplicate.

In vitro generation of cytotoxic effector cells

Cytotoxic effector cells were generated in 24-well, flat bottom tissue culture plates (Costar, Cambridge, MA) as previously described (Arora and Shearer 1982). In brief, responding spleen cells (5×10^6, not treated with ACK) from individual animals were co-cultured with 2000 rad-irradiated (^{137}Cs source, Gammator Isomedix, Parsipany, NJ) allogeneic BALB/c stimulator spleen cells (2×10^6) that had been freed of erythrocytes by ACK treatment. The culture plates were incubated at 37°C in 95% air/5% CO_2 for 5 days.

Cytotoxicity assay

Effector cells were tested for cytotoxicity by using a ^{51}Cr release assay (Arora and Shearer 1982) as follows: Cell dilutions (100 μl aliquots) were added to

microtiter wells (Linbro Chemical Co.). Target cells were 48 h Con-A-stimulated blast spleen cells that were labeled with 200 μCi/ml of ^{51}Cr (Na_2CrO_4, New England Nuclear, Boston, MA) and washed three times in HBSS. After counting, cells were added (100 μl aliquots) in triplicate to microtiter wells containing effector 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. The incubations were terminated by centrifugation for 5 min at 800 rpm, and the supernatants collected (Titertek Supernatant Collection System, Skatron, Sterling, VA). The radioactivity in the supernatant was measured in a Beckman Auto Gamma Counter (Beckman Instruments, Fullterton, CA). Samples were assayed in triplicate and the percentage of cell lysis determined (Arora and Shearer 1981) by the equation:

$$\% \text{ specific lysis} = \frac{\text{Exp}_{\text{cpm}} - \text{Con}_{\text{cpm}}}{\text{Max}_{\text{cpm}} - \text{Spont}_{\text{cpm}}} \times 100$$

where Exp_{cpm} was the number of counts released by effector cells cultured with allogeneic stimulating cells and Con_{cpm}, the number of counts released by effector cells cultured with autologous cells; Max_{cpm} was the number of counts released from 100 μl of target cells in the presence of a solution of 2.5% Triton-X detergent, and $\text{Spont}_{\text{cpm}}$ was the number of counts released from 100 μl of target cells in medium alone.

Results

Effect of FG 7142 on mitogen-stimulated T cell responses

Table 1 summarizes mitogen-stimulated T cell responses in splenic cultures of rats and mice injected with a single dose of FG 7142. The results are presented as both the cpm of [^{3}H]thymidine incorporated in the presence and absence of either Con-A or PHA and as the ratio of lymphocyte proliferation in the presence and absence (the 'stimulation index') of these mitogens. A statistically significant inhibition ($P < 0.02$) of both Con-A- and PHA-stimulated lymphocyte proliferation was observed in animals administered FG 7142. In four independent experiments, suppression of Con-A- (0.5 and 1 μg/well) stimulated lymphocyte proliferation ranged from 39.6 to 90.5% (74.3 ± 6%), while PHA (5 and 10 μg/well) stimulated lymphocyte proliferation was suppressed from 19.5 to 72.3% (52.4 ± 7%).

Blockade of FG 7142-induced immunosuppression by Ro 15-1788

Table 2 summarizes the effects of the benzodiazepine receptor antagonist Ro 15-1788 administered 30 min prior to injection of FG 7142. The spleens from these rats were removed 24 h after the last injection and mitogen-stimulated T cell responses determined as described (Materials and methods). While no differences in [^{3}H]thymidine incorporation were observed in the absence of mitogen, both Con-A (74.8%, $P < 0.05$) and PHA (74.1%, $P < 0.01$) stimulated lymphocyte proliferation were significantly inhibited in FG 7142-treated rats (Table 2). Ro 15-1788 pretreat-

TABLE 1

EFFECTS OF FG 7142 ON MITOGEN-STIMULATED T CELL RESPONSES

In individual experiments, rats (I–III) or mice (IV) were treated with FG 7142 or an equal volume of vehicle. The animals were killed by decapitation 24 h later and the mitogen-stimulated T cell proliferation determined in spleen cell cultures as described in Materials and methods. cpm are the mean ± SEM of five animals per group. The % suppression was estimated from the mean cpm in vehicle- and FG 7142-treated animals in the presence of mitogens. Administration of FG 7142 produced a statistically significant reduction ($P < 0.02$, paired t-test) in both Con-A- and PHA-stimulated T cell proliferation when the SI was compared in spleen cells from vehicle- and FG 7142-treated animals (four experiments) at each concentration of mitogen.

Expt.	Treatment	[^{3}H]TdR incorporation								
		None	Con-A				PHA			
		cpm	0.5		1.0		5.0		10	
			cpm	SI/% Supp.	cpm	SI/% Supp.	cpm	SI/% Supp.	cpm	SI/% Supp.
I	Vehicle	1266 ± 86	4051 ± 277	3.2	22001 ± 3837	17.4	26561 ± 1674	21.0	15445 ± 1000	12.2
	FG 7142	1361 ± 92	2450 ± 104	1.8/39.6	2093 ± 182	1.5/90.5	10820 ± 1002	7.9/59.3	10344 ± 465	7.6/33.1
II	Vehicle	1708 ± 92	10760 ± 1005	6.3	18690 ± 1155	11.2	20316 ± 1401	12.1	7857 ± 194	4.6
	FG 7142	2750 ± 481	3025 ± 215	1.2/71.9	3719 ± 1448	1.4/80.2	6871 ± 1088	2.5/66.2	6325 ± 150	2.3/19.5
III	Vehicle	1744 ± 142	119464 ± 11645	68.5	128026 ± 27050	71.5	16150 ± 2337	9.4	16045 ± 1504	9.2
	FG 7142	1169 ± 130	17353 ± 2404	15/85.4	10092 ± 3668	9.4/92.2	4486 ± 1125	3.7/72.3	6897 ± 253	5.9/57.1
IV (Mice)	Vehicle	982 ± 124	51948 ± 2993	52.9	65674 ± 4376	71.5	ND		14968 ± 933	16.1
	FG 7142	696 ± 24	16078 ± 1414	23.1/69.1	22924 ± 1829	33.1/65.1	ND		5952 ± 368	8.6/59.6

ND, not determined.

TABLE 2

BLOCKADE OF FG 7142-INDUCED IMMUNOSUPPRESSION BY THE BENZODIAZEPINE RECEPTOR ANTAGONIST, Ro 15-1788

Rats were injected with Ro 15-1788 (10 mg/kg) or an equal volume of vehicle 30 min prior to administration of FG 7142 (10 mg/kg) or vehicle. The animals were killed by decapitation 24 h later and the mitogen-stimulated T cell proliferation determined in spleen cell cultures as described in Materials and methods. Values represent the mean ± SEM of [^{3}H]thymidine incorporated. Four animals were used in each group, and each concentration of mitogen was assayed in quadruplicate. Statistical analyses were performed on the cpm of [^{3}H]thymidine incorporated by analysis of variance followed by Duncan's multiple range test.

Treatment	[^{3}H]TdR incorporation				
	None	Con-A		PHA	
	cpm	cpm (SI)	% Suppression	cpm (SI)	% Suppression
Vehicle/Vehicle	585 ± 107	215 216 ± 70 117 (348.2)		9182 ± 2459 (15.5)	
Vehicle/FG 7142	537 ± 46	54 196 ± 5766 * (101.1)	74.8	2379 ± 212 ** (4.5)	74.1
Ro 15-1788/Vehicle	555 ± 93	206 153 ± 34 047 (404.1)	4.2	8755 ± 1174 (16.3)	4.7
Ro 15-1788/FG 7142	535 ± 104	162 344 ± 21 353 (355.5)	24.6	4389 ± 796 * (9.2)	52.2

* $P < 0.05$; ** $P < 0.01$ compared with vehicle-treated animals.

ment restored Con-A-stimulated lymphocyte proliferation to values which were not significantly different from vehicle-treated animals, and reduced FG 7142-induced suppression of PHA-stimulated lymphocyte proliferation by ~ 30% (Table 2).

Suppression of CTL generation by β-carbolines

In another study designed to assess the effect of benzodiazepine receptor inverse agonists on immune function, mice were treated with a single dose of either DMCM or FG 7142. 24 h later, their spleens were removed and allogeneic cytotoxic T lymphocyte (CTL) activity was generated in vitro against BALB/c spleen cells (Materials and methods). A significant suppression of CTL activity ($P < 0.01$) was observed in mice treated with either DMCM or FG 7142 compared to animals that received only vehicle injections (Fig. 1).

Discussion

These studies demonstrate that administration of benzodiazepine receptor 'inverse agonists' elicit a profound suppression of the immune response in rodents, manifest as an inhibition of both mitogen-stimulated lymphocyte proliferation and

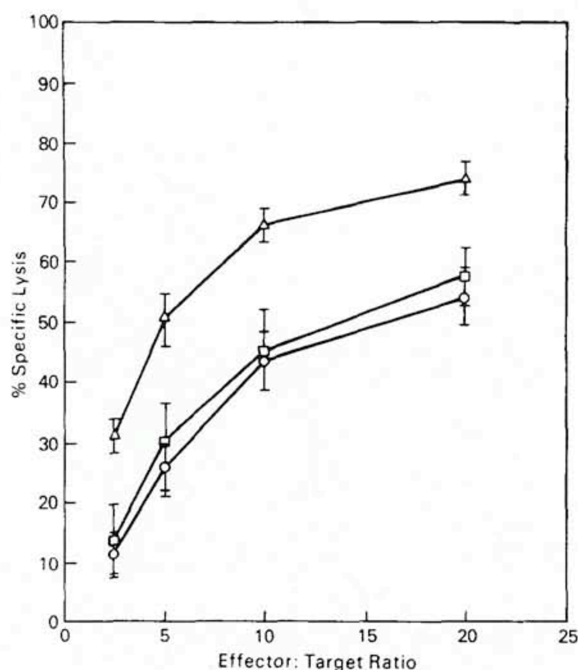

Fig. 1. Spleen cells from NFR/N mice injected 24 h, earlier with vehicle (△), FG 7142 (25 mg/kg) (□), or DMCM (3 mg/kg) (○), were cultured against allogeneic BALB/c spleen cells. Cytotoxic effector cell activity was tested on BALB/c spleen cells (48 h) Con-A blasts labeled with ^{51}Cr. Spontaneous release: 22%. Each value represents mean ± SEM of five animals. $P < 0.01$, vehicle versus DMCM- or FG 7142-treated animals, paired *t*-test.

CTL activity (Fig. 1; Tables 1 and 2). Benzodiazepine receptor inverse agonists (such as the β-carbolines used in this study) have been shown to bind with high affinities (Braestrup et al. 1983) to that population of benzodiazepine receptors linked to $GABA_A$ receptors and an associated chloride channel (Skolnick and Paul 1983). Occupation of benzodiazepine receptors by inverse agonists results in a reduction of GABA-gated chloride currents through neuronal membranes (Chan and Farb 1985). The pharmacological actions produced by such compounds may be best described as 'opposite' to the benzodiazepines; these actions include convulsions, increased sleep latency, and a syndrome resembling 'fear' or 'anxiety' (Skolnick and Paul 1983). Although the mechanism by which benzodiazepine receptor inverse agonists, such as DMCM and FG 7142 suppress T cell function is unknown, addition of either compound (1–100 μM) to T cell cultures does not inhibit mitogen-stimulated T cell proliferation (data not shown), which suggests these compounds are not (directly) toxic. Comparable viable cell counts of spleens from β-carboline- and vehicle-treated animals (data not shown) would support this hypothesis.

The doses of DMCM and FG 7142 used in the present study have been shown to elicit psychopharmacologic effects in rodents that are suggestive of 'fear' or 'anxiety'

(Stephens and Kehr 1983; Pellow and File 1985). Both the specificity of these compounds for benzodiazepine receptors in the central nervous system (CNS) (Braestrup et al. 1983) and the ability of the benzodiazepine receptor antagonist Ro 15-1788 to partially antagonize the effects of FG 7142 (Table 2) strongly suggest that the immunosuppression produced by these β-carbolines is initiated at benzodiazepine receptors in the CNS. The inability of Ro 15-1788 to fully antagonize the effects of FG 7142 (Table 2) may be due to the relatively short half-life of this compound in the rodents ($t_{1/2}$ = 16 min in rat brain; 15 min in plasma) (Lister et al. 1984). Since 'anxiogenic' β-carbolines increase sympathetic activity and stimulate hypothalamic-pituitary-adrenal axis (Ninan et al. 1982; Dorow et al. 1983; Insel et al. 1984), the immunosuppression observed could be mediated, either wholly or in part, by an action on either system (Williams et al. 1981; Felten et al. 1984; Bellanti 1985).

These findings support the hypothesis that there is a causal relationship between certain forms of stress and modulation of the immune function, and suggest that the CNS pathways subserved by benzodiazepine receptors may be important in the neural modulation of immunity. Recently, Costa and colleagues have purified and sequenced an ~ 11 kDa protein ('diazepam binding inhibitor', DBI) (Guidotti et al. 1983) and an 18 amino acid fragment of this protein ('ODN') (Ferrero et al. 1986) that bind to benzodiazepine receptors with moderate to low affinities (IC_{50} ~ 1–5 μM). These compounds elicit both 'proconflict' effects (Guidotti et al. 1983; Ferrero et al. 1984) and are convulsants, actions that are similar to those obtained with the β-carbolines used in the present study. Thus, it is possible that the immunosuppression produced by administration of β-carbolines could mimic the actions of endogenously occurring compounds. This hypothesis merits further investigation. Whether the same pathways subserved by benzodiazepine receptors are also responsible for the effects on immune function produced by environmental and psychosocial stressors (Ader 1981; Tecoma and Huey 1985) is unknown, but the availability of specific, high affinity ligands of the benzodiazepine receptor will prove useful in answering these questions.

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