ATTENUATION OF THE EFFECT OF PROGESTERONE AND 4'-CHLORDIAZEPAM ON STRESS-INDUCED IMMUNE RESPONSES BY BICUCULLINE

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Abstract : The present study investigates the effect of progesterone, a pregnane precursor of neurosteroids, and 4'-chlordiazepam (4'-CD), a specific ligand for mitochondrial diazepam binding inhibitor receptor (MDR) involved in neurosteroidogenesis, on restraint stress (RS)-induced modulation of humoral and cell-mediated immune responses. RS produced a significant reduction in anti-sheep red blood cells (SRBC) antibody titre, a measure of humoral immune response, and % leucocyte migration inhibition (LMI) and foot-pad thickness test, measures of cell-mediated immune responses. These effects of RS on immune responses were effectively blocked by pretreating the animals with progesterone (10 mg/kg, sc) or 4'-CD (0.5 mg/kg, sc) administered just before subjecting the animal to RS. The effect of both progesterone and 4'-CD on RS-induced immune modulation was significantly attenuated by bicuculline (2 mg/kg, ip) but not by flumazenil (10 mg/kg, ip). Unlike its effect on RS-induced immune responsiveness, progesterone (5, 10 mg/kg, sc) when administered to non-stressed animals produced a significant suppression of both humoral and cell-mediated immune responses which was not reversed by bicuculline. However, 4'-CD failed to modulate immune response in naïve non-stressed animals. These results suggest that progesterone and 4'-CD affect stress-induced immune responses by modulating GABA-ergic mechanism. However, GABA-A receptor system does not appear to be involved in progesterone-induced immunosuppression in nonstressed animals.

Key words: stress   humoral immune response   neurosteroids
cell-mediated immune response   GABA-A receptors

INTRODUCTION

It is well known that stress can affect various aspects of the immune response and may modulate the course and outcome of pregnancy and infectious, neoplastic and autoimmune diseases (1–5). Immune system and central nervous system (CNS) can

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influence each other in a reciprocal manner and receptors for various hormones and neurotransmitters present on the lymphocytes allow functional interaction between the CNS and the immune system (6–13).

Benzodiazepine (BZD)-GABA-A receptor-chloride channel complex is reportedly involved in the physiological regulation of many stress responses including gastric ulceration (14, 15), corticosterone levels (15, 16) and immune responsiveness (15, 17–19). Several steroid compounds synthesized *de novo* in the brain are known as neurosteroids (20). Important neurosteroids characterized in the CNS include progesterone, allopregnanolone (5α-pregnan-3 α-ol-20-one; AP), tetrahydrodeoxycorticosterone (5 α-pregnan-3 α-21 diol-20-one; THDOC), dehydroepiandrosterone and pregnenalone sulfate (20). A pathway for neurosteroidogenesis has been delineated in the brain and a role of mitochondrial diazepam binding inhibitor receptor (MDR) in the neurosteroid biosynthesis has been demonstrated (21, 22). Neurosteroids like AP and THDOC are reported to be positive allosteric modulators of the GABA-A receptors (23) and are shown to exhibit GABA-A receptor mediated antistress and anxiolytic activities (24, 25). BZDs such as diazepam are positive allosteric modulators of GABA-A receptors and have been shown to modulate the effect of restraint stress (RS) on humoral as well as cell-mediated immune responses (18, 19). The present study investigates the effect of progesterone, a pregnane precursor of neurosteroids like AP and THDOC (26) and 4'-chordiazepam (4'-CD), a high affinity MDR ligand (27) on RS-induced modulation of humoral and cell-mediated immune responses. Effect of progesterone and 4'-CD have also been studied in naïve non-stressed animals.

METHODS

Animals

The study was carried out in male Wistar rats (180–200 g) and Swiss albino mice (25–30 g) obtained from the Central Animal House of the University College of Medical Sciences, Delhi. They were housed in natural light and dark cycles and temperature (22 ± 2°C) controlled conditions. The feed (Pellet diet, Golden Feeds, Delhi) and water were available *ad libitum*, except during the periods of stress. The protocol of the study was approved by the Institutional Animal Ethics Committee and the care of the animals was as per the 'Guidelines for the Care and Use of Animals in Scientific Research' prepared by the Indian National Science Academy, New Delhi.

Drugs

Progesterone, 4'-Chlordiazepam (4'-CD), Bicuculline methiodide (Sigma, St Louis, USA) and Flumazenil (Hoffman La Roche, New Jersey, USA) were used in the study. Progesterone, 4'-CD and flumazenil were dispersed in 1% Tween 80 and diluted with saline while bicuculline methiodide was dissolved in distilled water. Progesterone and 4'-CD were injected subcutaneously (sc) and flumazenil and bicuculline were given by intraperitoneal (ip) injection. The doses of the drugs used were selected on the basis of pilot experiments and data available in literature (18, 24, 28, 29). In non-stressed

animals two doses (progesterone 5, 10 mg/ kg and 4'-CD 0.25, 0.5 mg/kg) were used while in stressed animals effect of the effective or higher dose was studied.

Stress procedure

For inducing stress rats were placed in Plexiglass restrainers (INCO, Ambala) at room temperature $(22 \pm 2°C)$ for 24 h. Previous work done in our lab has shown that 24 h of RS at room temperature produced much more immunosuppression as compared to 6 h of RS at room temperature or 3 h of cold restraint stress (18, 19). Hence 24 h of RS was used in rats to study drug effect. Mice were placed in round pipe mouse restrainers for inducing stress. In mice 6 h or 24 h RS produced marked mortality while 1 h RS every day for 5 days produced significant immunosuppression without mortality. Hence, this protocol was used for studying drug effect in mice.

Humoral immune response

Experiment in stressed animals : The rats were sensitized with sheep red blood cells (SRBC; 0.5×10^9 cells/ml/100 g, ip) on day 0. On day 7, they were injected with the same dose of antigen again (booster dose) and divided into different groups. One group was not subjected to RS but was deprived of food and water and kept in home cage. This group acted as 'no-stress' (NS) control. The other groups were treated with vehicle, progesterone (10 mg/kg), 4'-CD (0.5 mg/kg), progesterone (10 mg/kg) + flumazenil (10 mg/kg), progesterone (10 mg/kg) + bicuculline (2 mg/ kg), 4'-CD (0.5 mg/kg) + flumazenil (10 mg/ kg) or 4'-CD + bicuculline and then subjected

to RS in Plexiglass restrainers. After 24 h all the animals were lightly anaesthetized with ether and blood was collected from the retroorbital plexus. The serum was separated and haemagglutination titre was estimated as follows : 2-fold dilutions (0.025 ml) of sera were made in the microtitre plates with saline. To each well 0.025 ml of 1% (V/V) SRBC was added. The plates were incubated at 37°C for 1 h and then observed for haemagglutination. The highest dilution giving haemagglutination was taken as the antibody titre and expressed in a graded manner, the minimum dilution (1/2) being ranked as 1 and median value of different groups were compared by statistical analysis.

Experiment in non-stressed animals : The animals were sensitized with SRBC as above on day 0. From day 1–6 they were treated with vehicle, progesterone (5, 10 mg/kg), 4'-CD (0.25, 0.5 mg/kg) or progesterone (10 mg/kg) + bicuculline (2 mg/kg). On day 7 all the animals were bled from retroorbital plexus, serum was separated and haemagglutination titre estimated as above.

Cell-mediated immune response

Footpad thickness test :

Experiment in stressed animals : Mice were immunized with SRBC (1×10^8 cells, sc) on day 0. The animals then received vehicle, progesterone (10 mg/kg), 4'-CD (0.5 mg/kg), progesterons (10 mg/kg) + flumazenil (10 mg/ kg) progesterone (10 mg/kg) + bicuculline (2 mg/kg), 4'-CD (0.5 mg/kg) + flumazenil (10 mg/kg) or 4'-CD (0.5 mg/kg) + bicuculline (2 mg/kg) and then subjected individually to RS for 1 h everyday in round pipe mouse

restrainers from day 1–5 at room temperature ($22 \pm 2°C$). One group was not subjected to RS and acted as 'NS' control. On the 5th day, animals in all the groups were challenged with 1×10^8 SRBC, sc in the right hind paw, whereas normal saline was injected in the left hind paw. Increase in footpad thickness was measured 24 h after the challenge by fluid displacement method using mercury Plethysmograph. A mark was made on both the hind paws (right and left) just above the tibiotarsal junction. The paws were dipped in the mercury column up to the fixed mark and paw volume was measured by noting the mercury displacement. The difference in the volume of right hind paw (test) and left hind paw (control) was calculated and compared between the control and drug treated groups.

Experiment in non-stressed animals : Animals were sensitized with SRBC (1×10^8 cell, sc) as above. They were administered vehicle, progesterone (5, 10 mg/kg), 4'-CD (0.25, 0.5 mg/kg) or progesterone (10 mg/kg) + bicuculline (2 mg/kg) from day 1–5. On 5th day, as above all the animals were challenged with SRBC injected sc in the right hind paw and increase in footpad thickness was measured 24 h later.

Leucocyte migration inhibition (LMI) test :

Experiment in stressed animals : The rats were sensitized with 0.5 ml egg albumin (25 mg/ml) along with 0.5 ml complete Freund's adjuvant given sc on day 0. Animals in one group were not subjected to RS and acted as 'no stress' (NS) control. However, like

stressed animals they were food and water deprived on the days of stress. Animals in other groups were subjected to RS for 24 h at room temperature ($22 \pm 2°C$) in Plexiglass restrainers on day +1 and +13 (first and 13th day after the day of sensitization, i.e. day 0). Vehicle, progesterone (10 mg/kg), 4'-CD (0.5 mg/kg), progesterone (10 mg/kg) + flumazenil (10 mg/kg), 4'-CD (0.5 mg/kg) + flumazenil (10 mg/kg), progesterone (10 mg/kg) + bicuculline (2 mg/kg, ip) or 4'-CD (0.5 mg/kg) + bicuculline (2 mg/kg) was administered just prior to subjecting the animals to RS on day +1 and +13. During the period of stress the animals were food and water deprived. On the 14th day all the animals were anaesthetized with ether and the chest was opened. About 5–6 ml of blood was withdrawn in heparinized syringe by cardiac puncture and LMI test was performed (19).

Experiment in non-stressed animals : Animals were sensitized as above with egg albumin and complete Freund's adjuvant on day 0. On day 1–13, they were administered vehicle, progesterone (5, 10 mg/kg) or progesterone (10 mg/kg) + bicuculline (2 mg/kg). On day 14, LMI test was performed.

Statistical analysis

The data of anti-SRBC antibody litre were analysed using Mann Whitney 'U' test, while Student's 't' test and Chi-square test were used for analysing the data of foot pad thickness and LMI tests, respectively. A 'P' value of <0.05 was taken as the level of significance.

RESULTS

Humoral immune response

RS for 24 h at room temperature produced a significant reduction in anti-SRBC antibody titre. The antibody titre decreased from 8.0 (6–9) [median value (range)] in the 'NS' control group to 4.0 (3–7) in the stressed group (P<0.01). Pretreating the animals with progesterone (P<0.01) or 4'-CD (P<0.05) produced a significant attenuation of the effect of RS on anti-SRBC antibody titre. The effect of progesterone as well as 4'-CD on RS-induced immunomodulation was significantly (P<0.01) antagonised by bicuculline but not by flumazenil (Table I).

Administration of progesterone to non-stressed animals produced a dose-dependent suppression of anti-SRBC antibody titre (Table III). However, unlike its effect in

TABLE I : Effect of restraint stress (RS) and its modulation by progesterone and 4'-chlordiazepam on humoral immune response.

Treatment (mg/kg)	Anti-SRBC antibody titre Median value (range)
NS (control)	8.0 (6-9)
RS	4.0 (3-7)**[a]
Prog (10) + RS	7.5 (6-8)*[b]
4'-CD (0.5) + RS	7.0 (6-8)*[b]
Flum (10) + Prog (10) + RS	7.0 (7-8)
Flum (10) + 4'-CD (10) + RS	7.0 (6-8)
Bicu (2) + Prog (10) + RS	4.0 (3-5)**[C]
Bicu (2) + 4'-CD (0.5) + RS	4.0 (3-5)**[C]

n = 6
*P<0.05, **P<0.01 (Mann Whitney U test)
a. Compared to NS group; b. Compared to RS group; c. Compared to respective drug treated group
Flum = Flumazenil; Bicu = Bicuculline; Prog = Progesterone; 4'-CD = 4'-chlordiazepam

TABLE II : Effect of restraint stress (RS) and its modulation by progesterone and 4'-chlordiazepam on cell-mediated immune responses.

Treatment (mg/kg)	Foot pad thickness test (change in paw volume (mm))[††] (Mean±SEM)	% LMI[#]
NS (control)	0.076±0.002	54.00
RS	0.036±0.002*[a]	15.29*[a]
Prog (10) + RS	0.067±0.003*[b]	51.51*[b]
4'-CD (0.5) + RS	0.071±0.003*[b]	47.06*[b]
Flum (10) + Prog (10) + RS	0.069±0.003	49.02
Flum (10) + 4'-CD (0.5) + RS	0.070±0.003	52.10
Bicu (2) + Prog (10) + RS	0.044±0.002*[c]	22.31*[C]
Bicu (2) + 4'-CD (0.5) + RS	0.043±0.003*[c]	27.22*[C]

n = 6–8
*P<0.01
[††]Students 't' test; [#]Chi-square test
a. Compared to NS group; b. Compared to RS group; c. Compared to respective drug treated group
Flum = Flumazenil; Bicu = Bicuculline; Prog = Progesterone; 4'-CD = 4'-chlordiazepam

stressed animals 4'-CD failed to modulate haemaglutination titre in non-stressed rats. Further the immunosuppressive effect of progesterone was not blocked by bicuculline (Table III).

Cell-mediated immune response

Similar to the effect on humoral immune response, RS produced a significant suppression of cell-mediated immunity, the footpad thickness response as well as %LMI were significantly (P<0.001) reduced as compared to NS group. Pretreating the animals with progesterone or 4'-CD prior to stress procedure attenuated the effect of RS on both footpad thickness and % LMI. Like humoral immune response the effects of progesterone and 4'-CD on RS-induced

TABLE III : Effect of progesterone on humoral and cell-mediated immune response in non-stressed animals.

Treatment (mg/kg)	Humoral immune response Anti-SRBC antibody titre Median value (range)	Cell-mediated immune response	
		Food pad thickness test (change in paw volume (mm)) (Mean±SEM)	% LMI
Vehicle	6.5 (6-8)	0.076±0.002	54.00
Progesterone (5)	6.0 (5-7)	0.06±0.003	41.54*
Progesterone (10)	3.5 (3-5)*	0.038±0.002**	21.00**
Bicuculline (2) + Progesterone (10)	4.0 (3-5)	0.04±0.003**	27.40**
4'-chlordiazepam (0.25)	6.0 (5-7)	0.068±0.004	–
4'-chlordiazepam (0.5)	6.0 (5-8)	0.07±0.005	–

n = 6–8
*P<0.01; **P<0.001 (Vs. Vehicle-treated group)

modulation of cell-mediated immune responses were significantly antagonised by bicuculline but not by flumazenil (Table II).

Unlike its effect on RS-induced cell-mediated immune responses, progesterone when administered to non-stressed animals produced suppression of both footpad thickness response and %LMI. Like humoral immune response, this immunosuppressive effect of progesterone was not attenuated by bicuculline. Similar to its effect on anti-SRBC antibody titre 4'-CD in non-stressed animals did not modify the studied parameter of cell-mediated immune responsiveness (Table III).

DISCUSSION

Results of the present study show that RS produced suppression of both humoral and cell-mediated immune responses. This is in consonance with the results of earlier reports which also showed that RS suppressed both humoral and cell-mediated immune responses (18, 19). Pretreating the animals with progesterone or 4'-CD prior to stress procedure attenuated the effect of RS on immune parameters. Progesterone is a pregnane precursor of neurosteroids and is metabolised to allopregnanolone (AP) and tetrahydrodeoxycorticosterone (THDOC) in the neuronal glia of the brain (26). 4'-CD is a high affinity MDR ligand that stimulates mitochondrial neurosteroid biosyntheses (27). AP and THDOC are among the most potent of the known neurosteroids active at GABA-A receptors and have affinities greater than benzodiazepines (23, 30). BZD-GABA-A receptor complex is a multisubunit ligand gated ion channel. GABA binding increases Cl flux and this response can be modified by several class of drugs including BZDs, barbiturates and neurosteroids (23, 31). BZDs which produce their effect by augmenting GABA-A receptor mediated chloride ion conductance have been shown to antagonise RS-induced suppression of immune response (18, 19). AP and THDOC are also positive allosteric modulators of GABA (23). Hence, the observed immunomodulatory effect of progesterone and 4'-CD on RS-induced immunosuppression appears to be mediated by GABA-A chloride channel complex. This

explanation gains further credences from the fact that the effect of progesterone and 4'-CD were antagonised by bicuculline, a specific GABA-A receptor antagonist. Unlike bicuculline, flumazenil, a specific central BZD receptor antagonist (32) failed to modulate the effect of progesterone and 4'-CD, indicating thereby that neurosteroids are acting at a site other than the BZD binding sites on the GABA-A chloride channel complex. This observation corroborates the findings of other workers who also reported that effects of neurosteroids are blocked by GABA-A receptor antagonists (bicuculline, picrotoxin) but not by flumazenil (24).

In the present study, unlike its effect in the stressed animals progesterone when administered to naïve non-stressed animals produced suppression of both humoral and cell-mediated immune responses. Other workers have also reported progesterone-induced immunosuppression (33–39). Progesterone administration was observed to reduce lymphocyte proliferation and expression of activation markers CD25 and CD69 on lymphocytes. It also decreased the production of cytokines like interferon-gamma and down regulated co-stimulatory proliferative activity of IL-1 alpha/beta (33, 34). Progesterone has also been shown to exert a dose dependent inhibitory effect on the frequency of immunoglobulin-secretory cells in peripheral blood mononuclear cell culture by modulating CD8+ T cells which are known to control B cells secretory activity (35). Spleen and blood lymphocytes are reported to express progesterone receptors whose concentration is increased greatly in presence of progesterone (36). Progesterone binding with these receptors results in the synthesis of a mediator protein named the progesterone-induced blocking factor that inhibits antibody synthesis and cellular immunoreactions and proliferations (36, 37). The immunosuppressive effect of progesterone observed in the present study was not antagonised by bicuculline indicating thereby that this effect was not mediated via GABA-A receptors. Further, it is reported that progesterone per se is devoid of binding to GABA-A receptors (40). Hence, the immunosuppressive effect of progesterone in non-stressed animals appears to be mediated by its action on progresterone receptors present on lymphocytes.

It has been suggested that during stress there is release of an endogenous stress mediator in the body which decreases the GABA function so as to produce the stress syndrome (41, 42). An 11-k dalton protein has been identified in the brain having affinity for BZD receptors, which has anxiety producing effects (43). The reduction in GABA function during stress may make GABA-A chloride channel complex more susceptible to the antistress effects of neurosteroids resulting in attenuation of immunosuppressive effects of RS by progesterone and 4'-CD. However, in naïve non-stressed animals progesterone may act directly on progesterone receptors present on lymphocytes leading to suppression of the immune system. Failure of 4'-CD, a high affinity ligand for MDR that stimulates neurosteroidogenesis to modulate immune responsiveness in non-stressed animals further supports the contention that it is during stress that GABA-A chloride channel complex becomes more susceptible to the anti-stress effects of neurosteroids.

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