Rats were subjected to unpredictable chronic stress (UCS), which was composed of 3 cycles of 7 kinds of stress for 21 days. Rats given UCS exhibited a depressive state in behavioral tests such as emergence tests and forced swim tests. Administration of cyclosporine-A (CsA), an immunosuppressive drug, gave rise to antidepressant effect in rats under the UCS, but not in stress-free rats. In other words, CsA shortened both the latency time in emergence tests and the immobility time in forced swim tests in rats given UCS. Analysis of brain tissue by HPLC revealed that CsA caused a significant increase in NE, 5-HT, and 5-HIAA levels in the cortex of UCS treated rats, but treatment with either UCS or CsA alone resulted in the opposite effect. Comparing the data of monoamines and their metabolites in the brain, cascades may be different between CsA and imipramine, although both of them showed antidepressive effect in behavioral tests.

Key words: cyclosporine, behavior, neurotransmitter, imipramine, depressive

Introduction

Cyclosporine-A (CsA) is a drug commonly employed for its immunosuppressive effects in organ transplantation, and is currently being investigated in clinical trials for a wide range of autoimmune diseases. In addition to the immunosuppressive effect, other adverse effects of CsA so far reported are encephalopathy, hyperactivity, hypertension and neurotoxicity. In rats, injection of high-dose of CsA (20 mg/kg) for 2 weeks caused EEG abnormalities. Borlongan reported that long-term administration of CsA (for 21 and 32 days) significantly increased nocturnal spontaneous and amphetamine-induced hyperactivity in rats. In an experiment of short-term injection (for 3 days) in rats, CsA induced a decrease of ambulatory activity in the open field and increased defecation. Some investigators have reported that CsA increases serotonin (5-HT) levels in the brain of rats and mice. It is also reported that CsA may have direct beneficial effects on dopaminergic neurons and dopamine-mediated behaviors. However, it is not yet known if CsA really interacts with neurotransmitters in the brain. In practice, the concentration of CsA in cerebrospinal fluid (CSF) was low after the systemic injection in rats and humans.

In the present study, we injected CsA intraperitoneally (i.p.) into rats under unpredictable chronic stress (UCS) and investigated the effect on the central nervous system (CNS) in terms of behavioral parameters and amount of neurotransmitters in the brain, as compared with an anti-depressant, imipramine. As
behavioral parameters, we examined the spontaneous locomotor activity by using "ANIMEX" machine, and latency, frequency and emergence time by emergence tests, and immobility time by forced swim tests. Emergence tests that provides a measure of fear-induced inhibition of exploratory activity is usually used to see the effect of anti-anxiety drugs\textsuperscript{13,14}. Forced swim tests are used to see the effect of anti-depressant drugs\textsuperscript{15,16}.

\textbf{Materials and Methods}

\textbf{Subjects}

Six-week-old male Fischer 344 rats (Charles River Japan, Japan) were used in the present study. The rats were maintained on a 12 hr light-dark cycle (light on 8:00-20:00) in a temperature controlled colony room (\(22^\circ\text{C}\)). Rats had free access to food and water. The study was approved by the Guidelines for Animal Experimentation, Tokyo Medical and Dental University (Japan).

\textbf{Drugs}

The immunosuppressive drug, cyclosporine-A (CsA; Sandimmune\textsuperscript{5}, Sandoz Pharmaceuticals Co., DE), was dissolved in peanut oil (10 mg/ml). The tricyclic antidepressant, imipramine (Hydrochloride Sigma Ultra\textsuperscript{6}, Sigma Chemical Co., MO), was dissolved in 0.9\% HCL water (10 mg/ml).

\textbf{Unpredictable chronic stress (UCS)}

Rats were subjected to 3 cycles of 7 kinds of stress for 21 days, one stress episode per day\textsuperscript{17}. Seven kinds of stress were given as follows: shaking for 15 min, water deprivation for 24 hr, cold stress at \(0^\circ\text{C}\) for 30 min, restraint for 1 hr, heat stress at \(40^\circ\text{C}\) for 30 min, food deprivation for 24 hr and tail pinch for 5 min.

\textbf{Behavioral analysis}

Rats were divided into 10 groups (\(N = 9\)). Five groups were given stress (S groups) for 21 days as mentioned above and the other five were not given stress (NS groups).

All rats were subjected to behavioral tests for 3 days (pretests) before the stress exposure and the drug treatment. The tests were comprised of locomotion tests on the 1st day, emergence tests on the 2nd day and forced swim tests on the 3rd day. After the pretest, rats of five S-groups were subjected to UCS as mentioned above. Five pairs of S- and NS-groups were given imipramine (S-I/NS-I) intraperitoneally (i.p.), vehicle (peanut oil) alone (S-CsA0/NS-CsA0) or 3 doses (2.5, 5.0, 10.0 mg/kg body weight) of CsA (S-CsA2.5/NS-CsA2.5, S-CsA5/NS-CsA5, S-CsA10/NS-CsA10), once per day for 21 days in parallel with the schedule of UCS. From the next day of the last day of the exposure to UCS and/or drug treatment, all rats underwent behavioral tests similar to the pretests.

Locomotion was evaluated by ANIMEX (FADAD Co., Sweden).

Emergence tests were performed in an apparatus consisting of two parts, dark starting (33 \(\times\) 23 \(\times\) 25 cm) and a transparent-emergence-compartment (20 \(\times\) 12 \(\times\) 15 cm) connected through a gate (8.1 cm). The three behavioral parameters recorded in this apparatus were latency, frequency and emergence time. (A) Latency; time interval (seconds) between gate-opening and the first emergence (defined as emergence when the whole body of the animal, except for the tail, entered the emergence cage). When rats did not emerge, latency was scored as 300 seconds (total test duration). (B) Frequency; frequency that rats passed the gate. (C) Emergence time: total time (seconds) which rats spent in the emergence compartment.

In forced swim tests, rats were individually introduced into a transparent glass cylinder (50 cm in height and 17 cm in diameter) filled with tap water (\(25^\circ\text{C}\)), and immobility time (seconds spent floating without moving) was scored. After the test, rats were wiped with a towel and warmed in a incubator at \(37^\circ\text{C}\) for 10 min.

\textbf{Determination of monoamines and their metabolites in the brain}

Three days after the last day of drug treatment and/or exposure to UCS, the brains were removed in 3 pairs of S- and NS-groups given i.p. imipramine (S-I/NS-I), peanut oil alone (S-CsA0/NS-CsA0) and CsA (5 mg/kg body weight per day) (S-CsA5/NS-CsA5). The frontal cortex and hippocampus were dissected on ice, rapidly frozen in liquid nitrogen and stored at -80\(^\circ\text{C}\) until use. The frozen tissue was homogenized in 0.1N perchloric acid (PCA) containing 1 mM ascorbic acid (0.6 times of the wet weight). After centrifugation (10,000 rpm, 15 min, 0\(^\circ\text{C}\)), a part of supernatant was used for protein assay and the other 10 \(\mu\text{l}\) aliquot was adjusted to pH 3 with 2M sodium acetate and injected into a high-performance liquid chromatography system with an electrochemical detector (HPLC-ECD) for the determination of NE (nor epinephrine), DA (dopamine), DOPAC (dihydroxyphenylacetic acid), HVA (homovanillnic acid), 5-HT (serotonin), 5-HIAA (S-
hydroxyindoleacetic acid). The electrode potential was set at 750mV vs. Ag/AgCl reference electrode. The mobile phase used for the assay was 0.1 M phosphate buffer containing 1.7% methanol and 190 mg/L SOS (1-octanslfon acid)-NA, pH 3.5, 25°C. The HPLC system consisted of a reverse phase column (Eicompak SC-5ODS, 3.0mm x 150mm, Eicom Co., Japan) and an electrochemical detector ECD-100 (Eicom Co., Japan). The quantity of protein in the tissue sample was measured by Bio-Kinetics Reader EL 312e Microplate (Bio-tec Instruments, Winooski, VT) using a protein assay kit (Bio Rad, Japan). The amount of monoamines and metabolites was shown as the ratio to total protein (ng/mg).

Flow cytometry
Thymocytes and splenocytes (1 x 10^6) were stained with following monoclonal antibodies for 30 min at room temperature: PE-labeled OX-35 (anti-CD4) and FITC-labeled OX-8 (anti-CD8). Stained cells were resuspended in PBS containing 2% FBS and analyzed by FACScan (Becton Dickinson, Mountain View, CA) using CELL QUEST software. All antibodies were purchased from Pharmingen (Los Angeles, CA).

Statistics
All data were presented as mean ± S.E.M. Results were analyzed using ANOVA and Fisher’s PLSD. Pearson’s product-moment correlation coefficient (r²) was also used to see the dose dependent effect.

Results

Effect of CsA and unpredictable chronic stress (UCS) on the weight of body and adrenal glands
Rats given UCS exhibited a significant decrease of body weight (Fig. 1A). When comparing NS groups, a higher dose of CsA caused a decrease in body weight (NS-CsA5, NS-CsA10) as compared with NS-CsA0 (F = 20.79, p < 0.01) (r² = 0.44). On the contrary, UCS gave rise to a significant increase of adrenal weight (Fig. 1B). In comparing the S groups, those treated with higher doses of CsA showed the greatest adrenal gland weight increase (Fig.1B) (F = 25.91, p < 0.01) (r² = 0.62).

Effect of CsA and unpredictable chronic stress (UCS) on the behavior of rats
Rats were divided into two groups; S groups with stress and NS groups without stress. Before administra-

Effect of UCS and CsA on monoamines and metabolites in the frontal cortex
Administration of CsA resulted in an opposite effect among rats treated with UCS and those without UCS
Fig. 1. Effect of UCS and/or cyclosporine-A (CsA)/imipramine (I) on the body weight (A), weight of adrenal glands (B), locomotion tests (C), emergence tests (D), and the immobility time in forced swim tests (E).

Emergence tests (D) is composed of 3 parameters. Latency(a): Time interval (sec) between cage gate opening and the first emergence. The emergence is defined as entrance of the whole body of the animals, except for the tail, into the emergence cage. When rats did not emerge, latency was scored as 300 sec (total test duration). Frequency (b): frequency of emergence. Emergence time (c): Total time (sec) a rat spent in the emergence cage.

Pretests (Pre): The average of values in all groups before the treatment and exposure to stress (N = 45). The results are displayed as means ± S.E.M (N = 9). NS: no stress. S: stress. I: imipramine injected (10mg/kg). CsA0: no CsA injected. CsA2.5: CsA injected (2.5mg/kg). CsA5: CsA injected (5mg/kg). CsA10: CsA injected (10mg/kg). The level of significance: *p < 0.05, **p < 0.01 (vs CsA0); #p < 0.05, ##p < 0.01 (NS group vs S group).
Fig. 2. Monoamines and their metabolites in frontal cortex. The results are displayed as means ± S.E.M. Pretest: the average values in all groups; NS: no stress. S: stress; I: imipramine injected (10 mg/kg). CsA0: no CsA injected. CsA5: CsA injected (5 mg/kg).
The level of significance: *p < 0.05, **p < 0.01 (vs CsA0); *p < 0.05 (NS group vs S group).
A significant increase in NE (F = 17.00, 
P < 0.01) and 5-HT (F=4.25, 
P < 0.05) and a trend of increase in DA, DOPAC, HVA and 5-HIAA were observed in the rats given UCS, but the effects were opposite in rats not treated with UCS (Fig. 2).

**Effect of UCS and CsA on the immune system**

Either stress, CsA or both caused a decrease in thymus, subpopulations of thymocytes, and splenic T cells and B cells (Table-1, 2).

**Discussion**

In the present study, we examined the effect of cyclosporine A (CsA) on the behavior of rats under unpredictable chronic stress (UCS) by using Fischer 344 rats. Fischer 344 rats are known to be sensitive to stress than Sprague-Dawley rats. In the locomotion test, initially reported by Holland and Weldon was employed to access locomotive activity and emotional reactivity. Previous studies reported that UCS suppressed locomotion of animals. Other studies, however, reported that restraint stress or
injection of corticosterone releasing hormone induced an increase of locomotion. In the present study, we found that the locomotion did not change in rats given only stress or low doses (2.5 and 5 mg/kg) of CsA, but decreased in rats given a higher dose of CsA (10 mg/kg) both in no stress or stress groups. CsA at 10 mg/kg might be toxic dose in Fischer 344 rats, although LD50 = 14 mg/kg in male Wister rats.

The emergence test was introduced by Crawley and Goodwin to assess the anxiolytic effect of benzodiazepines in terms of exploratory activity. Stress induced anxious behavior. Rodriguez employed the emergence test to see the effect of UCS on the behavior. Using rats, they reported that the stress increased emergence latency, and reduced the frequency of emergences and total time in the emergence cage. In the present study, stress alone gave a trend of increase in the tests for latency, although statistically not significant. Administration of either CsA or imipramine resulted in a significant decrease in emergence latency in rats under stress. The findings suggested that both CsA and imipramine showed anxiolytic effects under stress. However, such an anxiolytic effect of CsA was not evident in the tests for the frequency of emergences and emergence time. On the contrary, imipramine showed a significant increase in emergence time, indicating anxiolytic effect.

Schechter and Chance employed the forced swim test to assess the depressive condition of mice by measuring the time of characteristic immobile posture; i.e., elongation of immobilization time in mice in depressive conditions. In our experiments, the immobility time increased in UCS treated rats and was shortened by administration of CsA or imipramine. It is of interest to note that the effect of CsA and imipramine was seen only in the groups given UCS, but not in those without UCS.

Furthermore, we found that UCS treatment resulted in a significant decrease (NE, 5-HT, 5-HIAA) or a trend of decrease (DA, DOPAC, HVA) in the content of neurotransmitters in the frontal cortex of rats. The administration of CsA was effective in increasing the amounts of neurotransmitters in UCS treated rats; i.e., a significant increase in NE, 5-HT and 5-HIAA and a trend of increase in DA, DOPAC and HVA. On the contrary, imipramine did not influence the content of monoamines and their metabolites in the behavioral tests.

Frontal cortex is susceptible to stress. An increased level of DA and NE in the frontal cortex is associated with the enhanced locomotor activity. In major depression, however, NE, DA, 5-HT and their metabolites are known to decrease in the CNS. Stress for more than 5 days gives rise to a decrease of NE (hypothalamus, hippocampus), DA (nucleus accumbens) and 5-HT (cortex). Taken together these findings indicate that, like imipramine, CsA may act as an anti-depressant, although the cascade of CsA in the brain may be different from that of imipramine.

It is interesting to note that stress may cause loosening of the blood-brain-barrier (BBB). Immobilization stress, heat stress, fever, infection, liver damage and bone marrow transplantation can increase permeability of the BBB.

In our preliminary study using mice, the permeability of the BBB increased during heat stress (40°C, 15 min) in caudate putamen and fimbria hippocampus (data in possession of the authors). Therefore, we are planning a new experiment to see how the stress influences the permeability of CsA in rats.

CsA alone, when injected at a larger dose, can affect the brain. Our findings, however, suggests the possibility that even a smaller dose of CsA might enter into the brain through the loosened BBB due to stress and influence neurotransmitters in the brain.

It is also possible to say that CsA suppresses IL-1, IL-2 and TNF-alpha in peripheral and prevented the decrease of 5-HT and NE which causes depression.

The present study suggests that CsA acts like an anti-depressant in behavioral tests and may also up-regulate the content of monoamines in the brain of rats under stress. This rodent model would be suitable for further elucidation of the mechanisms of the effects of CsA on the CNS.

Acknowledgment

We gratefully acknowledge the support by Eicom Co., Japan.

References

4. Fazakerley JK, Webb HE. Cyclosporine, blood-brain barrier,


