[原著] Studies on the effect of noise stress on immune response of mice

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ABSTRACT

The immune responses of mice exposed to noise stress 12 hours daily for 18 days was investigated. Immune responses were estimated by antibody production to sheep red blood cell (SRBC), lymphoproliferation response and relative change of lymphocyte subsets in the thymus and spleen, with reference to plasma corticosteroid and catecholamine levels. A decrease in antibody response to SRBC, with an increase of plasma corticosteroid levels and a decrease in total thymocyte number, were observed in mice challenged with SRBC on the 7th day of noise exposure. The antibody response, however, was enhanced over control levels in mice challenged on the 18th day, with a decrease in corticosteroid levels and an increase in thymocyte number. The catecholamine level increased considerably after onset of the noise stress. A decrease of CD 4 /CD 8 double negative cells, with a relative increase of CD 4 /CD 8 double positive cells, was also observed in the thymus on day 3. Both $CD\,4^+$ and $CD\,8^+$ single positive cells also decreased significantly in the thymus. These results suggest that depression of T-cell differentiation lineage in the thymus occurred in the stressed mice. The relative number of $CD\,4^+/CD8^-$ (helper type) lymphocytes decreased in the spleen of mice on the 3rd day of the noise stress. Changes in the relative population of the lymphocyte subset, however, recovered to nearly normal levels on the 18th day of the experiment. These results indicate that, although the immune responses were depressed tentatively in the early stages of noise exposure, noise stress might essentially enhance the immune responsiveness of mice. *Ryukyu Med. J., 17(4)189~197, 1997*

Key words: noise stress, antibody response, lymphocyte subset, corticosteroid, T-cell lineage

INTRODUCTION

The immune system maintains long term self-integrity by removing non-self and, as is demonstrated in recent studies, involves an effector system regulated by the neuroendocrine system. Conversely, the immune response is also known to have a possible influence upon the emotions and actions of humans through various cytokine actions. Interest is now centered on the internal "cross-talk" between the immune system and neuroendocrine system[1-3].

It is well known that stress may lead to an incresed susceptibility to pathogens such as bacteria and virus and to the risk of development of carcinoma via suppression of immune function[4,5]. A number of reports have indicated that many kinds of stress such as burn injury[6], high pressure[7], surgical operation[8] and immobilization of the body[9] have a high intensity or very prolonged suppressive effect on the immune response. It is believed that these stressors activate the hypophysis-adrenal cortex which enhances the secretion of immunosuppressive hormones such as the adrenocorticotrophic hormone (ACTH) and glucocorticoids. On the other hand, some stressors, such as pain and isolation, have been known to strengthen the immune response[10-12]. Therefore, it is considered that different stressors may induce different effects on the immune system and that the effect of stress on the immune system may be variable according to the intensity and duration of stress exposure.

Noise is also known to be a severe physical stressor for living organisms. However, there is little information concerning the effect of noise stress on the immune system. In a previous study, the authors reported that noise stress has an enhancing effect on the immune response in rats exposed to noise for 4 hours every day for 18 days[13]. In the present study, the effect of noise stress on the immune system was further examined in mice which received noise stimulation for 12 hours daily for 18 days.

MATERIALS AND METHODS

Animals

BALB/c male mice, purchased from the Kyudo Animal Supply Center Co. Ltd., Kumamoto, Japan, were used throughout the experiments. They were 6 weeks old and weighed about 20 grams at the start of the experiments. Two or three mice were kept in each cage (30 ×36×16cm) to avoid the stress of overpopulation or isolation. All mice were housed at a constant temperature of 25 ± 2 ℃ and a humidity level of 60± 5 %, and exposed to a light-dark regimen (lights on from 0700-1800hours). All mice were given proprietary brand food and water ad libitum. A total of 100 mice divided into 20 groups of 5 mice each were used in the present study.

Noise stress

The mice were subjected to white noise at about 100dB using a noise signal generator (SF-05, Rion Company Ltd., Tokyo, Japan). The noise stress was administered daily for 12 hours from 19:00 to 7:00hours, at the height of their diurnal activity cycle. Mice in a quiet room, in which the noise level was about 40dB (A), were used as controls. The control and experimental mice were sacrificed at the same time.

Measurement of plasma corticosteroid and catecholaminne

The mice were sacrificed on days 7 and 18 after onset of noise stimulation. The sera were obtained 1 hour after the end of the noise exposure on each experimental day.

The corticosteroid level in the sera was measured by fluorescence polarization immunoassay (FPIA)[14] in which fluorescent-labelled corticosteroids (tracer) and unlabeled corticosteroids (analyte) in the sample serum reacted with antibodies specific for corticosteroid. In this method, the tracer competes for antibody binding sites with the concentration of the analyte. The greater the concentration of analyte, the larger the fraction of the tracer that is unbound. When linearly polarized light is used to excite the tracer and when the tracer is bound to the antibody, the fluorophore is still highly polarized upon emission. On the other hand, when the tracer is free, its rotation is much greater and the emitted light is depolarized. Standards with known amounts of corticosteroids are read and the polarization recorded. When an unknown is read, its concentration is estimated by interpolating between standards.

Measurement of plasma catecholamines, such as adrenaline, noradrenaline and dopamine, was performed using a three-column system of high-performance liquid chromatography (HPLC)[15]. After treatment with precolumns, samples were applied on an analytical column (TSKgel Catecholpak; Tosoh Co. Ltd., Yamaguchi Japan) and catecholamines were eluted separately. The elutes were then allowed to react with fluorogenic reagent and the fluorescence intensity of the eluates which was converted to diphenylethylene-diamine derivatives was measured at 470 nm.

For these procedures, specialized automated fluorescence polarization analyzer and HPLC analyzer are necessary; the samples were therefore consigned to Kitazato Biochemical Laboratories, Tokyo, for the measurement.

Peripheral blood neutrophil count

Collection of peripheral blood for hematological examination was done on every 3rd day after the noise exposure was begun. Mice were bled in the same manner on each occasion. All mice were bled during the morning hours to avoid artifacts due to diurnal variation. Prior to bleeding, mice were lightly anesthetized with ether, and the tail vein was then severed with a lancet. The first few drops of blood were wiped away and a smear was made on a clean slide glass. The smear was fixed in 100% methanol for 5 min and stained with Giemsa. Differential leucocyte counts were made of at least 200 nucleated cells on the basis of morphology and the number of neutrophil was expressed as a percentage of the total.

Thymus size

The mice were sacrificed on days 7 and 18 of noise stimulation and the thymus removed into Eagle MEM medium (Flow Lab., Mcleam, USA). Adherent fat and connective tissues were removed carefully from the thymus and single cell suspensions were prepared by forcing the thymus through a fine mesh stainless steel sieve (#200). After washing once, cells were suspended in an appropriate volume of MEM medium and mononuclear cells were stained by adding Turk solution to the suspension. The cell concentration was calculated using an improved 'Neubauer' haemocytometer chamber and the total cell number in the thymus was calculated from the cell concentration and total volume of the suspension.

Measurement of anti-sheep red blood cell (SRBC) plaque-forming cells (PFC)

The mice were challenged with 0.2 ml of 5 % SRBC suspension on days 7 and 18 and antibody forming cells against SRBC were assessed 7 days after the challenge.

The method used for assaying IgG PFC was essentially as described by Cunningham and Szenberg (1968)[16] with certain modifications made in this laboratory. The spleen was removed and single cell suspensions in MEM medium were prepared by gently sieving through a 200-mesh stainless steel screen. After washing once, a 0.75% Tris-ammonium chloride solution (pH 7.65) was added to the cell pellet to hemolyze the contaminated erythrocytes. The cells were washed again three times and resuspended in RPMI 1640 medium (Flow Lab.USA) supplemented with 10mM HEPES (Gibco Lab., Grand Island, NY, USA) and 5 % fetal calf serum (FCS) to make approximately 1×10^7

viable cells/ml. The number of IgM plaques were counted as direct plaques. The cell suspension (0.4 ml) was mixed at room temperature with 0.05 ml of 50% SRBC suspension and with 0.05 ml of 1:4 diluted guinea-pig serum as complement absorbed with SRBC in an ice bath. The mixture was again placed in the glass slide chamber (Takahashi Giken Glass Co. Ltd., Tokyo). The chamber was sealed with paraffin to incubate at 37℃ for 45 min. The hemolytic plaques were counted under a microscope and IgM PFC/10^8 spleen cells were calculated from the number of PFC and total cell number applied in the chamber. The indirect plaques for IgG PFC were further enumerated by adding rabbit anti-mouse IgG serum at a concentration of 1:100 to the above mixture; This was previously determined optimal to reveal IgG-PFC. The number of IgG-PFC was expressed as the number of indirect PFC minus the number of direct PFC.

Mitogenic responses of spleen cells

Lymphoproliferative responses were performed by a standard microculture technique with flat-bottomed 96-well microculture plates. Spleens were removed from mice on days 7 and 18 of noise stimulation and spleen cell suspension was prepared as above in culture medium (RPMI 1640) containing 10 mM HEPES and 5% FCS. The cell suspension was adjusted to 2.5×10^6 and 0.2 ml (5×10^5 cells) of it was applied to each well with or without 10 μl of the following mitogens: phytohemagglutinin-purified (PHA-P, 12.5 μg/ml; Difco Lab., Detroit, USA), concanavalin A (Con A, 2.5 μg/ml; Sigma Chem. Comp., St Louis, USA) and lipopolysaccharide (LPS, 50 μg/ml; Difco Lab.). All cultures were incubated in triplicate at 47℃ in a 5 % CO_2 atmosphere for 3 days in a CO_2 incubator (LNA-111DH, Tabai Espec Co. Ltd., Tokyo Japan). On the final day of the cultivation, cells were pulse labeled with 0.1 MBq of tritiated methylthymidin (^{3}H-TdR, specific activity: 74.0 MBq/mmol; New England Nuclear, Boston, USA) for the last 7 hours and harvested by filtration through glass filter paper with a multiple sample harvester apparatus (Lab Mash; Labo Science, Tokyo). The radioactivity (counts per minute: cpm) of each sample was measured in a liquid scintillation counter (Aloka LSC-900; Aloka Co., Tokyo Japan). The results were expressed as stimulation index (SI); the ratio between cpm in mitogen-stimulated culture to that in unstimulated culture.

Cell surface marker analysis of thymocytes and spleen cells

Single cell suspensions from thymus and spleen were prepared from mice on days 3 and 18 of noise exposure, and lymphocyte subpopulation were identified by phenotypic analyses of surface markers linked to specialized function. For detection of the surface marker antigens, FITC- or phycoerythrin (PE) -conjugated monoclonal antibodies against CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD45RA (Ra3-6B2), $\alpha\beta$-TcR (h57-597) and $\gamma\delta$-TcR (GL3) were used in the present study. The monoclonal antibodies were all from PharMingen, San Diego, USA. Two-color staining was performed to identify the subpopulations. Briefly, 1×10^6 cells in PBS were stained with 10 μl of the appropriate mixture of the above labeled monoclonal antibodies in the Eppendorf tubes at 4℃ for 20 min in the dark. The cells were washed once by cold PBS and suspended in PBS to filtrate through nylon mesh. The stained cells were analyzed for their fluorescence intensity by flow cytometry on a Spectum III (Ortho Diagnostic System, Cytoron, USA). The subpopulations were measured on 1×10^4 cells for each sample.

Table 1 Plasma corticosteroid and catecholamines in noise stressed mice

	Duration of noise stress(days)		
	0	7	18
Corticosteroid (μg/dl)	1.7	4.0	0.9
Adrenaline (pg/ml)	734	1,880	4,790
Noradrenaline (pg/ml)	1,650	2,130	15,000
Dopamine (pg/ml)	462	235	16,600

The sera, 1.0ml each from five mice, were collected and pooled on days 0,7 and 18 of noise exposure and levels of plasma corticostroid and catecholamines were measured.

Statistics

Results were presented as means±SD and the statistical difference between control and experimental groups was analyzed using unpaired Student's T-test. The Stat View computer program (Abacus Concepys, Inc., Berkeley, USA) was employed to test the significance level among the groups and $P<0.05$ was considered significant.

RESULTS

Table 1 shows the concentration of serum corticosteroid and catecholamines (adrenaline, noradrenaline and dopamine) after noise exposure. The noise-stressed mice showed about 2.3 times increase of corticosteroid concentration against that of unstimulated control mice on day 7. The corticosteroid concentration, however, decreased to half that of control mice on day 18. The concentration of adrenaline and noradrenaline increased after exposure to noise and reached 6.5 times and 9.0 times those of controls, respectively. However, plasma dopamine level in the stimulated mice decreased slightly on day 7, but increased by about 36 times that of the control mice on day 18. The change of dopamine concentration showed a reverse in relation to that of the corticosteroid concentration in the stressed mice. Fig. 1

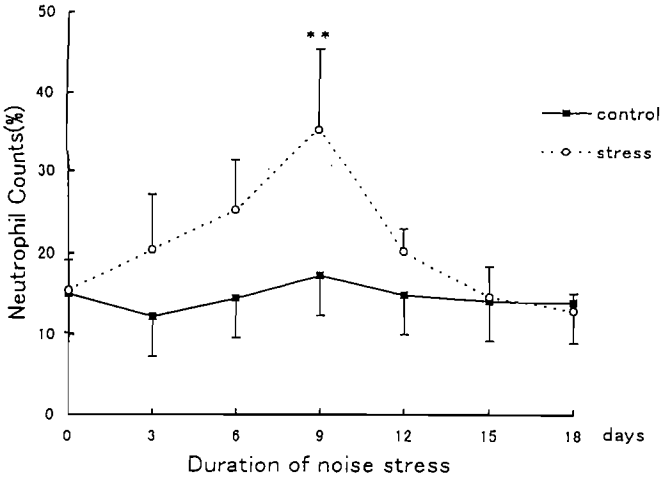

Fig. 1 Noise stress-induced effect on relative percentage of peripheral neutrophils in mice. Two groups of five mice were examined periodically every 3 days with or without noise.stimulation. Each point and vertical line represent mean±SD.
**Significantly different ($p<0.01$) from those of mice unstimulated.

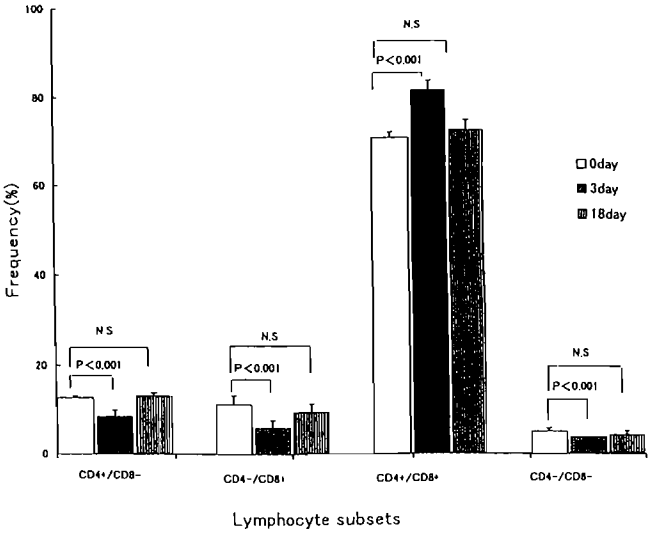

Fig. 3 Effect of noise stress on lymphocyte subsets in thymus of mice.
Each bar and vertical line represent the mean value± SD for each phenotypic cells for each group of five mice on days 0, 3 and 18 after stress exposure.

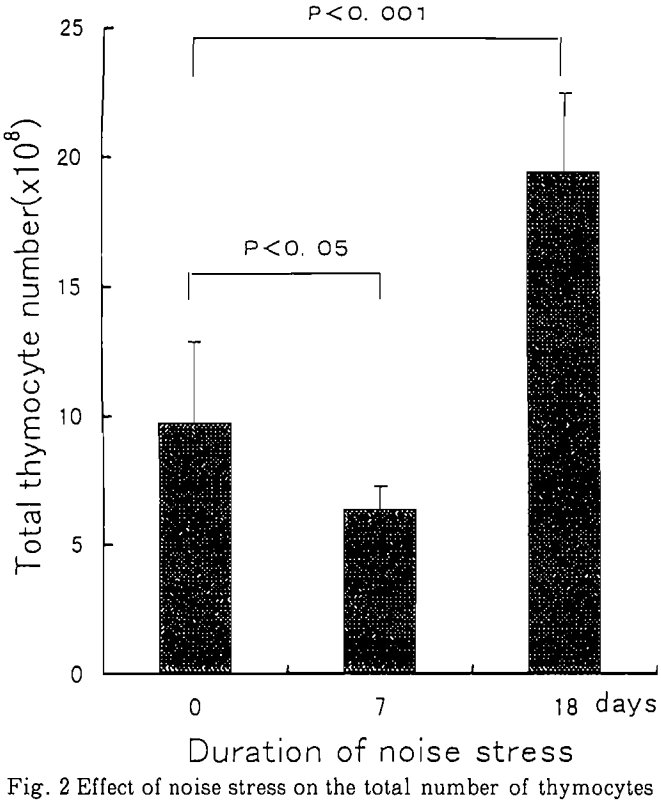

Fig. 2 Effect of noise stress on the total number of thymocytes in mice.
Each bar and vertical line represent the mean value±SD for each group of five mice on days 0, 7 and 18 after noise stimulation.

represents the kinetics of peripheral neutrophils in the stressed mice. The relative percentage of neutrophils increased gradually up to day 9 after noise exposure; by day 12, the neutrophil count had begun to decrease and on day 18 had returned to control level.

In Fig. 2, the total number of thymocytes of stressed mice on days 7 and 18 are compared with those of control mice. Total thymocytes showed a significant decrease on day 7, but had increased over the control number by day 18.

In the thymus, lymphocyte subsets for CD4 and CD8 were analyzed (Fig. 3). Directly after the onset of noise stimulation on day 3, the relative proportion of CD4/CD8 double negative thymocytes decreased significantly, while that of double positive thymocytes showed a significant increase. The thymocytes single positive for CD4 or CD8 also showed a relative decrease in the thymus. On the other hand, these changes in the relative proportion of thymocyte subsets returned to almost control levels on day 18.

A similar examination was performed on the spleens of the same mice for CD3, CD4, CD8, CD45RA and $\alpha\beta/\gamma\delta$ TcR phenotypes (Fig. 4). On day 3, both $CD3^+$ T cells and $CD45RA^+$ B cells increased in relative numbers, although the differences between the noise stimulated and unstimulated mice were not statistically significant. Among the $CD3^+$ T cells, the $CD4^+$ helper-type subset T cells showed a significant decrease in relative proportion, but it was not significantly different from the control mice for the $CD8^+$ suppressor-type T cell subset. In the same spleens, $\alpha\beta$ TcR positive cells showed a tendency to decrease after noise exposure but was not significantly different from that of control mice. On the contrary, although the proportion of $\gamma\delta$ TcR positive cells in the normal spleen is quite low, compared to the noise stimulated mice, it increased

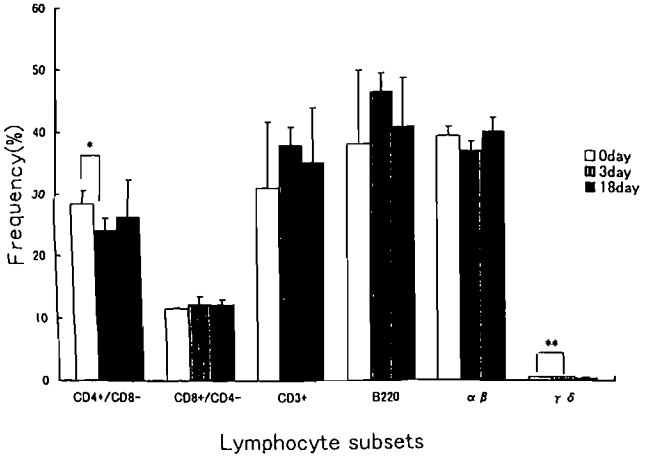

Fig. 4 Effect of noise stress on lymphocyte subsets in spleens of mice.
Each bar and vertical line represent the mean value+SD for each phenotypic cells for each group of five mice on days 0, 3 and 18 after stress exposure. $^{*}p<0.05$, $^{**}p<0.01$

significantly Representative patterns of the above results are shown in Fig.5. Two-color staining for CD4 and CD8 (Fig.5a) showed that the thymus of the stressed mice had increased levels of double positive cells and decreased levels of double negative and single positive subsets. An additional pattern (Fig.5b) represents an elevated level of γ δ TcR positive cells in the spleen, although α β TcR positive cells did not show a proportional change. These proportional changes in spleens, however, returned to normal levels on day 18, as was the case of thymocytes.

Spleen cells from stressed mice challenged with SRBC on day 7 of noise exposure showed decreased production of IgG PFC to SRBC, but it was considerably enhanced in mice challenged on day 18 of stress (Fig.6). The PFC responses showed about a 40% decrease in mice challenged on day 7 and a 35% increase in mice on day 18; neither difference, however, was statistically significant.

Figure 7 represents the levels of proliferative responses estimated by ^{3}H-thymidin uptake in stimulated spleen cell cultures. The spleen cells from stressed mice tended to have a reduced response to the PHA stimulant after exposure to noise stress. On the other hand, the response level of splenic cells from stressed mice did not differ from that of control mice in the response to the LPS stimulant on day 7, but it also decreased by day 18. However, these differences were not stastistically significant.

DISCUSSION

There have been many studies concerning the effect of noise stress on living organisms. The studies have not only been concerned with the physiological effect on the auditory organ but also on the sympathetic nervous system and endocrine systems[17-23]. From these

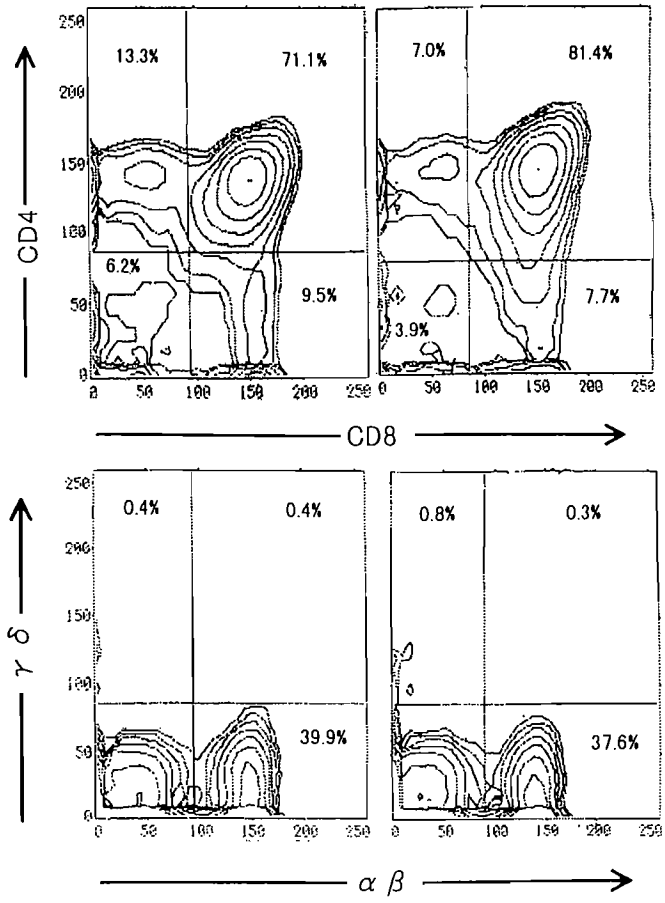

Fig. 5 FACS analysis on thymocytes and splenic lymphocytes before and after noise stimulation. Representative patterns by the two color analysis are shown, revealing relative increase of CD4/CD8 double positive cells in thymus (a) and relative increase of γ δ TcR positive cells in spleen (b) on day 3 after noise stimulation.

studies, it has been established that noise has a strong effect on the neuroendocrine system. However, from the viewpoint of cross-talk between the neuroendocrine system and the immune system, there have been very few investigations of the effect of noise stress on the immune system[24]. In a previous study, Freire-Garabal *et al.* (1995)[25] investigated the effects of buspirone on the immune system of mice exposed to a chronic auditory stressor and found that auditory stress did result in suppression of natural killer cell activity and also in a decrease of the in vivo and in vitro phagocytic activities of immune cells. In another study, where human subjects were exposed to a mental exercise of arithmetic plus a noise stressor, the results revealed that the stressor increased natural killer cell numbers and cytotoxicity, absolute numbers of $CD8^{+}$ T-lymphocytes, norepinephrine and epinephrine levels, heart rate, and blood pressure response[26]. In a previous report by the authors using rats exposed to noise stress for 4 hours daily, it was found that noise stress did not lead to suppression of the immune response but rather enhanced it[13]. In that

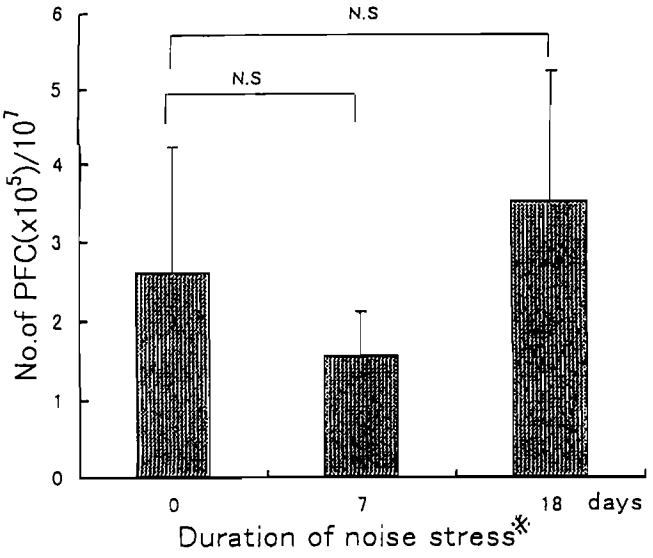

Fig. 6 Antibody response to SRBC under noise stimulation in mice.
*Duration of noise stress before SRBC challenge: Mice were challenged with SRBC on days 7 and 18, and PFC assay was performed 7 days after the SRBC challenge. Each bar and vertical line represent the mean PFC number ±SD for each group of five mice.

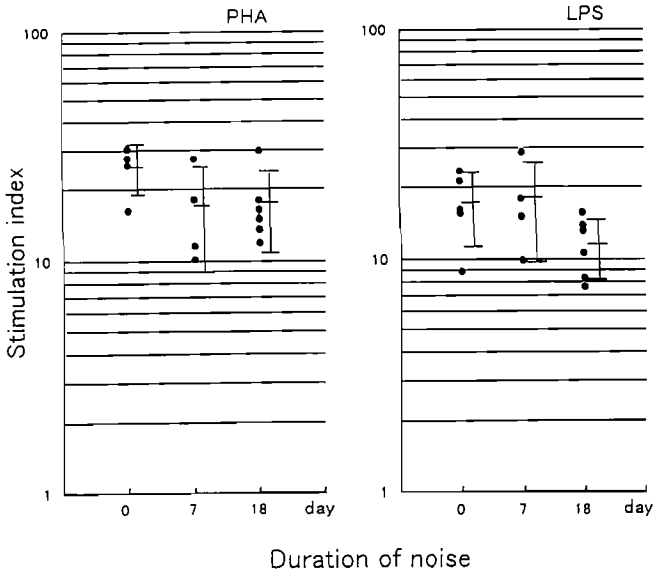

Fig. 7 Effect of noise stress on blastogenic responses of spleen cells of mice.
Each point represents the mean stimulation index of three replicate cultures. Horizontal and vertical lines represent the mean±SD for each group on days 0, 7 and 18 after noise stimulation.

study, however, the noise stress was probably not intense enough to suppress the immune response since rats were exposed to noise stimulation for only 4 hours daily. Therefore, in the present study the exposure time of noise stimulation to mice was prolonged to 12 hours daily, at night. The results showed a marked increase in corticoid hormone concentration, and an accompanying atrophy of the thymus, immediately after the noise exposure. A suppressive effect on the capacity to produce antibodies to SRBC was also observed in the same mice. These results indicate that noise stress may suppress the immune system. However, the suppressive effect was found to be transient. The corticoid hormone level decreased significantly in the following stage of noise stimulation, together with a significant increase of thymus size and the enhancement of antibody response. The data presented here extended previous observations concerning conditioned alteration of the immune responses to a noise stressor and indicated that living organisms might be able to react differentialy to external stimuli, which has been associated with different immunological consequences.

It is well known that the corticoid hormone has a strong suppressive effect on the immune response. There have also been many reports concerning the effect of noise stress on the adrenal cortex. In some of these reports, it has been suggested that noise stress enhances the function of the adrenal cortex by the secretion of corticoid hormone[27]. On the other hand, other studies have reported that noise stress has no effect or has a suppressive effect on the adrenal cortex function[28,29]. This difference in results may be explained by the fact that each study used a different sort of noise as well as different stimulation conditions, such as intensity of noise and duration of exposure. It has been well documented that stress, such as burn injury or immobilization, causes suppression of the immune response and is accompanied by elevated levels of plasma corticoid hormone and marked atrophy of the thymus[7,30-32]. This could be explained by the fact that the enhancement of the secretion of corticoid hormone induces apoptosis of thymocytes[33]. In the present study, a reverse relation was recognized between the level of corticoid hormone and the thymus size as esti mated by total cell number. On the other hand, it is known that some kinds of stress activate the hypothalamus-sympathetic nervous system and that the secretion of adrenaline is enhanced by the stimulation of the adrenal medulla. In these cases, atrophy of thymus does not occur and the immune response is enhanced. In this study, the plasma concentrations of adrenaline and noradrenaline in mice increased after exposure to noise stress. In the later stage of stress exposure, when antibody response recovered from a transient decrease, these concentrations increased up to about 6 and 9 times that of normal levels. In addition, the level of dopamine which is closely related to brain activity, slightly decreased in the early stage of noise exposure, but showed significant increase with time.

There have been many reports on the effect of noise stress on the circulatory system in which noise stimulation led to constriction of the blood vessels and the enhancement of secretion of plasma epinephrine. It is also known that some stressors increase the peripheral neutrophil population

via stimulation of the sympathetic nervous system[17,34,35]. This is because the immune function shifts to a direct defense system by neutrophils under the influence of the activated sympathetic nervous system. In the present study, the peripheral neutrophil population showed a transient increase immediately after the exposure of mice to noise. The authors, in a previous study, have shown a similar transient increase in neutrophil in rats after exposure to noise. Neutrophils increased transiently because of the tension in the sympathetic nervous system created by the noise. The enhanced adrenaline secretion due to the noise stress led to increased neutrophils via stimulation of the adrenergic receptor on their surface[36]. In the later stage of noise exposure, neutrophils returned to normal levels regardless of the plasma adrenaline and noradrenaline concentrations which were still maintained at high levels under the tension in the sympathetic nervous system. The reason is not clear but it may be hypothesized that the adrenaline response to α-adrenergic receptor shifted the response to the β-adrenergic receptor which affected the different cells. This may be the so-called acclimatization, and it could be explained that the mice adapted themselves quickly to noise stimulation. The increase in relative proportion of neutrophils implies a decrease in peripheral lymphocytes, which may relate to the suppression of the immune response in the initial stage of noise exposure. In this study, however, considering the fact that the ratio of neutrophils in peripheral blood is only about 10% in mouse and rat (which is considerably different from human beings whose neutrophils occupy about 60-70% of total peripheral leucocytes), the possibility that this transient increase of neutrophils affected the depressed antibody response was considered to be negligible. Indeed, in our previous study, although neutrophils in the peripheral blood in rats markedly increased, suppression of antibody production was not noticed.

When phenotypic subsets of thymocytes were compared between stressed and control mice, it was found that CD4/CD8 double negative immature cells decreased, while double positive cells increased proportionally. A significant decrease in single positive cells for CD4 or CD8 was also observed. The proportional changes of thymic cell subsets returned to almost normal condition in the later stage of the present experiment when mice recovered from transient suppression of antibody response, suggesting that the change in thymic cell subsets might have some relation to the suppressed immune response.

It is well known that the immature double negative cells derived from bone marrow differentiate to double positive cells and then to each single positive mature cell in the T-cell differentiation lineage in the thymus. In the differentiation process, almost all of the immature double positive cells die in the thymus, and the mature single positive cells can be distributed to the systemic lymphoid organs via the thymus medulla[37]. In addition to the significant decrease of the total number of thymocytes, the significant decrease in double negative and single positive cells might indicate that the stress suppressed T-cell differentiation in the thymus. These results coincide with the decrease in helper-type T lymphocytes positive for CD4 in the spleen which is a peripheral lymphoid organ. On the other hand, in the other studies using mice, it has been reported that cells developed during stress exposure exhibit a suppressive effect to antibody response[8,38]. These suppressive cells were phenotypically negative for L3T4, and were considered to be extrathymic T cells[28]. In our present study, the ratio of $\gamma\ \delta$-type TcR positive T-lymphocytes which is characterized as extrathymic T-lymphocytes[39] showed a significant increase in spleen cells from mice in the early stage of the noise stimulation when suppression of antibody response was noticed. However, it is not clear whether this type of cells has the same function of suppressing the immune responses.

From the above discussion we concluded that the noise stress in the present study has an essentially enhancing effect on the immune system, although a transient suppression of the immune response was observed in the acute period of noise stress. The effects of noise stress on the immune system were clearly related to the neuroendocrine system. As mentioned above, however, the effect of stress on the immune response may be affected to a great extent by the kinds of stressors and the duration of exposure to the stress. With regard to stressors that cause severe suffering such as pain and immobilization, it is predicated that the effect on the body and its adaptation to the stress will vary with the type of stress. In order to understand the effect of noise stress on the immune system, further intensive studies under different conditions should be carried out.

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