Stress and Immune Responses

I. Suppression of T Cell Function in Restraint-Stressed Mice

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Abstract—Effects of restraint stress on humoral immune responses were investigated in mice. Mice were restrained for 12 hours per day at nighttime and released at daytime for 2 consecutive days, either before or after sheep red blood cell (SRBC) immunization. The antibody response to SRBC was markedly suppressed in mice that were restrained before antigen injection. In contrast, the response was not significantly affected when the stress was loaded after immunization. Oral administration of 10 mg/kg diazepam prevented the stress-induced suppression of anti-SRBC antibody response. On the other hand, antibody responses to T cell-independent antigens such as trinitrophenylated (TNP)-Ficoll and TNP-lipopolysaccharide were not suppressed. These results suggest that the restraint stress causes dysfunction of T cell populations in mice.

Emotional stress can influence immune responses (1) and this has been shown experimentally in several circumstances (2–7). Many authors have reported the suppression of antibody responses by certain kinds of stressors. Vessay has reported that overcrowding stress developed significantly less precipitating antibody to bovine serum in mice (8). Hill and co-workers subjected monkeys to various 'psychological' stresses such as noise, light or loss of support after immunization with bovine serum albumin, and they observed lower antibody level in the stress group (9). More studies were conducted by Solomon (10). On the other hand, it has been also reported that development of cancer and autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus is caused by dysfunction of immune regulations (11, 12). The development of these diseases might be concerned

with stress loading in experimental animals (13–18). Therefore, it is interesting and important to elucidate the basic mechanisms underlying the stress-induced modulation of immune responses. However, the mechanisms of modulation are unknown.

Physical restraint has been one of the most popular stressors in experimental medicine, since it can be regulated easily and is reproducible (19–21). Restraint stress proved to be very effective in eliciting typical non-specific stress manifestations such as thymicolymphatic and splenic atrophy, leukocytosis, eosinopenia, lymphopenia, gastroduodenal ulcers and enlargement of the adrenals (22). Moreover, it has been reported that restraint stress increased susceptibility to simplex virus infection in resistant mice (14) and induced significant reduction of the antibody titre to Typhoid 'H' antigen in rats (23).

We found that restraint stress significantly suppressed the antibody response to sheep red blood cells (SRBC) in mice. In the present paper, we attempted to elucidate the mechanisms of stress-induced modulation of immune responses by employing restraint

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stress.

Materials and Methods

Animals: Female BALB/c mice (6 weeks old) were obtained from Japan Charles River Breeding Laboratories (Tokyo, Japan). All mice were maintained on MF pellets (Oriental Yeast Co., Ltd) and used when they were 9–12 weeks old.

Reagents: The reagents used in this work were as follows: SRBC, Nishinohon Sheep Farm (Fukuyama, Japan); lipopolysaccharide (LPS) (*E. coli*, 055: B5) and agar noble, Difco Laboratories (Detroit, MI); Ficoll 400, Pharmacia Fine Chemicals (Uppsala, Sweden); Eagle's minimum essential medium (MEM), Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan); monoclonal anti-Thy-1.2 antibody, Olac Co., Ltd. (Blackthorn, England); diazepam, Towa Yakuin (Osaka, Japan). SRBC were washed three times with sterile saline before use. Fresh serum of guinea pig was used as the complement source. Anti-mouse Ig serum was prepared in our laboratory by the repeated immunization of rabbits with purified BALB/c IgG fraction.

Stress procedure: Experimental mice were fixed in restraint cages for 12 hr per day at night (21:00–9:00) and placed in home cages for the remaining 12 hr with food and water ad libitum. The restraint cages were prepared in our laboratory according to the literature (24). Control animals were maintained in home cages from which food and water were removed for the period of stress loading of the counterparts. Usually mice were loaded with restraint stress for 2 consecutive days.

Immunization of antigens and assay of antibody-forming cells: Mice were immunized with 1×10^8 SRBC intravenously, 2 μ g trinitrophenylated (TNP)-Ficoll intravenously or 10 μ g TNP-LPS intraperitoneally. Four days later, antibody production was assayed by enumerating the number of hemolytic plaque-forming cells (PFC) by the method of Jerne and Nordin (25). TNP-conjugated Ficoll and LPS were prepared according to the methods of Haba and Hamaoka (26) and Rittenberg and Amkraut (27), respectively. Lightly conjugated TNP-SRBC were prepared as described by Rittenberg and Pratt (28) for

detecting anti-TNP PFC.

Determination of antibody titres in mouse serum: Antibody titre in mouse serum was determined by the hemagglutination procedure. Briefly, two-fold serial dilutions of sera from immunized mice were prepared by gelatin veronal-buffered saline in a 96 well microtitre plate (Tomi Seiko Co., Ltd., Tokyo). Twenty five μ l SRBC suspension (2×10^8 cells/ml) was added to 25 μ l of diluted serum and incubated at 37°C for 1 hr. The antibody titre of the serum is expressed as the reciprocal of the highest dilutions showing definite agglutination.

Analysis of T cell and B cell populations in spleen cells: Rabbit anti-mouse Ig serum or monoclonal anti-Thy-1.2 antibody was used to remove B cells or T cells, respectively. In brief, 1×10^7 spleen cells were incubated in 0.5 ml of MEM containing 1:2.5 anti-Ig serum or 1:500 anti-Thy-1.2 monoclonal antibody for 30 min at 4°C. Then the cells were washed twice with 3 ml MEM and resuspended in the original volume of MEM containing 1:8 volume of complement. After incubating for 30 min at 37°C, viable cells were counted by the trypan blue exclusion method.

Statistics: Statistical significance was calculated by Student's *t*-test. Differences were considered to be significant when probability (*P*) values < 0.05 were obtained.

Results

Antibody response to SRBC in restrained mice: In the first experiment, we investigated the effect of restraint stress on antibody response to SRBC, a T cell-dependent (TD) antigen. Mice were fixed for 2 days followed by immunization with SRBC. As shown in Fig. 1, the PFC response to SRBC in restrained mice was reduced to 30–60% of the control mice. Antibody titres in stressed mice were also decreased. In proportion to the duration of days of stress loading, the immune response was suppressed more severely (data not shown).

Thymus weight and the number of spleen cells in stressed mice was also reduced to about 50% and 65% of the control, respectively (Table 1).

In contrast, when mice were given the

Fig. 1. Suppression of the antibody response against SRBC in restrained mice. BALB/c mice were fixed in the restraint cage for 12 hr per day for 2 consecutive days, and then they were intravenously immunized with SRBC (1×10^8). The number of PFC in the spleen was determined 4 days after immunization. The results represent the mean $\pm$ S.D. of 4 animals. Significantly different from each control group: ** $P < 0.01$.

Table 1. Changes of body weight, thymus weight and the number of spleen cells in restrained mice

Experimental group	Body weight (g)	Thymus weight (mg)	The number of spleen cells ($\times 10^7$)
Exp. 1			
Control	21.3 $\pm$ 0.4	53.4 $\pm$ 8.1	14.3 $\pm$ 3.1
Stress	20.5 $\pm$ 1.2	23.0 $\pm$ 6.8**	10.5 $\pm$ 1.9*
Exp. 2			
Control	22.6 $\pm$ 0.4	48.7 $\pm$ 1.5	15.8 $\pm$ 0.5
Stress	21.1 $\pm$ 0.5	30.6 $\pm$ 7.8**	9.7 $\pm$ 1.3**

BALB/c mice were fixed in the restraint cage for 12 hr per day for 2 consecutive days. Body weight, thymus weight and the number of spleen cells were measured 4 days after the last stress loading. Results represent the mean $\pm$ S.D. of 4 animals. Significantly different from each control group: * $P < 0.05$, ** $P < 0.01$.

same stress after immunization with SRBC, the antibody response was not significantly affected. This result is shown in Table 2. It was found that only restraint stress before immunization significantly suppressed the antibody response.

Antibody responses against TNP-Ficoll and TNP-LPS in restrained mice: Effect of restraint stress on PFC responses against T cell-independent (TI) antigens such as TNP-Ficoll and TNP-LPS was investigated. Mice

were restrained on the same schedule as shown in Fig. 1 and then immunized with TNP-Ficoll or TNP-LPS. As illustrated in Fig. 2, the antibody responses to these antigens were not suppressed by restraint stress. The number of PFC per 10^6 spleen cells was slightly increased in contrast to the case of the anti-SRBC response.

Analysis of lymphocyte subpopulations in the spleen of restrained mice: As indicated in Table 1, the number of spleen cells in stressed

Table 2. Effect of restraint stress loaded after immunization on the direct PFC response against SRBC

Experimental group	PFC ($\times 10^3$)/spleen	PFC/ 10^6 spleen cells
Control	453 $\pm$ 47	2420 $\pm$ 222
Stress	373 $\pm$ 27	2648 $\pm$ 238

BALB/c mice were intravenously immunized with SRBC (1×10^8 cells/mouse) and then fixed in the restraint cage for 12 hr per day for 2 consecutive days. The number of PFC in the spleen was enumerated 4 days after immunization. The results represent the mean $\pm$ S.D. of 4 animals.

Fig. 2. Antibody responses against TNP-Ficoll and TNP-LPS in restrained mice. BALB/c mice were fixed in the restraint cage for 12 hr per day for 2 consecutive days, and then they were intravenously immunized with SRBC (1×10^8) or TNP-Ficoll (2 μ g) or intraperitoneally immunized with TNP-LPS (10 μ g). The number of PFC in the spleen was determined 4 days after immunization. The results represent the mean $\pm$ S.D. of 4 animals. Significantly different from each control group: * $P < 0.05$, ** $P < 0.01$.

mice was found to be reduced to about 65% of the control. In order to clarify which subpopulation of lymphocytes is more sensitive to stress stimulus, spleen cells from control and stressed mice were compared with regards to the proportion of T cells to B cells by treating spleen cells with anti-Ig serum or anti-Thy-1.2 antibody in the presence of complement. As indicated in Table 3, no significant differences were observed in the proportion of T cells to B cells between control and restrained mice. The result suggests that T cells and B cells have the same sensitivity to the stress in the

decrease of cell number.

Effect of diazepam on the suppression of PFC response against SRBC in restrained mice: Restraint-induced modulation of the immune reaction is probably mediated by the central nervous system (CNS). In order to ascertain this, anti-SRBC PFC response was examined in restrained mice that were orally administered with 10 mg/kg diazepam before stress loading. As shown in Fig. 3, administration of diazepam considerably recovered the suppression of immune response in stressed mice.

Table 3. Analysis of the cell population in spleens obtained from control and restrained mice

Experimental group	anti-Thy-1.2 antibody sensitive cells (%)*	anti-Ig antibody sensitive cells (%)*
Control	53.8±0.4	46.2±1.0
Stress	49.6±0.8	50.5±0.8

BALB/c mice were fixed in the restraint cage for 12 hr per day for 2 consecutive days. Cell populations were determined by the dye exclusion test after treatment of spleen cells with anti-Thy-1.2 or anti-Ig antibody and complement. The results represent the mean±S.D. of 4 animals. *: % of the whole spleen cell number.

Fig. 3. Effect of diazepam on the suppression of antibody response against SRBC in restrained mice. BALB/c mice were fixed in the restraint cage for 12 hr per day for 2 consecutive days, and then they were intravenously immunized with SRBC (1×10^8). Diazepam was orally administered daily for 3 days beginning one day before the start of stress loading. The number of PFC in the spleen was determined 4 days after immunization. The results represent the mean±S.D. of 4 animals. Significantly different from the control group: ** $P < 0.01$. Significantly different from the stress group: # $P < 0.05$.

Discussion

In the present paper, it was demonstrated that short-term and severe restraint stress prior to the immunization significantly suppressed anti-SRBC PFC response. Most of the studies about stress and immune reactions have shown the immunosuppressive effects of acute stress (2, 3, 5, 7, 23). Our data are also consistent with these

conclusions. For the present experiments, we chose 2 days for stress loading, since this treatment could induce significant immunosuppression without an appreciable effect on the body weight of mice. On the contrary, the stress loading after immunization had no suppressive effect on anti-SRBC PFC response, suggesting that spleen cells primed with antigen may become resistant to the suppressive effect of restraint stress.

Monjan and Collector have demonstrated that mitogen-induced blastformation of B cells and T cells were suppressed to the same extent by sound stress (29). The previous works, however, did not clarify the subpopulations of lymphocytes affected by stress loading in experimental animals (6, 30). We found that PFC response to a TD antigen of SRBC was suppressed by stress loading, but the response to TI antigens, TNP-Ficoll and TNP-LPS, were not suppressed. It has been reported that the antibody response to TNP-Ficoll requires macrophages (31, 32). Therefore, these results suggest that T cell functions are suppressed more heavily than functions of B cells and macrophages.

As shown in Fig. 2, the total number of PFC in the spleen against TNP-Ficoll or TNP-LPS were not decreased, but the number of PFC per 10^6 spleen cells was rather increased in contrast to the case of SRBC. In spite of the decrease in the absolute number of B cells in the spleen of stressed mice (Tables 1 and 3), PFC responses to these antigens were approximately the same as that of the control. As a plausible explanation for this phenomenon, we propose the following three points: (1) B cell populations that respond to TI antigens are not affected by stress procedures. (2) The functions of B cells are rather enhanced. (3) Stress sup-

presses the functions of suppressor T cells. It has been suggested that TI antigens can activate B cells without a stringent requirement for assistance from helper T cells, but were regulated by suppressor T cells in developing PFC responses (33, 34). Now in vitro experiments are in progress to elucidate the mechanisms of this phenomenon. Many questions about the mechanisms underlying stress-induced modulation of immune responses have been raised in these experiments. However, our data clearly demonstrated that restraint stress altered the antibody responses, depending on the timing of stress loading and suppressed helper T cell functions in mice.

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