Stress and Immune Responses

II. Identification of Stress-Sensitive Cells in Murine Spleen Cells

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Accepted January 6, 1986

Abstract—The influences of restraint stress on the functions of T cells, B cells and adherent cells in antibody responses were investigated. Antibody response against sheep red blood cells (SRBC), a T cell-dependent antigen, in cultured splenocytes from restrained mice was reduced to about 40–50% of that from the control mice. Addition of normal T cells to these cultures, however, restored the suppressed response. Moreover, helper T cell activities were lowered in restrained mice. On the other hand, suppressor T cell activities induced by both concanavalin A (Con A) and SRBC were significantly decreased in restrained mice. However, the antibody responses to T cell-independent antigens in stressed mice were approximately 40% higher than the control response. These enhancement were also observed in T cell-depleted splenocytes. Polyclonal antibody response induced by lipopolysaccharide (LPS) was increased in stressed mice. Antigen presenting cell activities were little influenced by restraint stress. Proliferative response to Con A, but not that to LPS, was suppressed in splenocytes from restrained mice. These results suggest that both helper and suppressor activities of T cells are suppressed, but B cell activity is rather enhanced in splenocytes from restrained mice.

Stress and its effects on immunological function, in both human and animal studies, have been a recent focus of psychosomatic medicine (1–5). However, the mechanisms of these interactions are unknown. Especially, influences of stress stimuli on immunocompetent cells still remain to be elucidated in antibody responses. In a previous paper, we investigated the effects of restraint stress on immune responses in mice and found that restraint stress caused suppression of the antibody responses to T cell-dependent (TD) antigens, but did not suppress the responses against T cell-independent (TI) antigens (6). For the

mechanisms of the diverse effects of restraint stress, we propose the following three hypotheses: (1) B cell populations that respond to TI antigens are essentially resistant against stress procedures. Recently, many authors reported the existence of different B cell subpopulations (7–10). (2) The functions of B cells are potentiated by restraint stress. (3) Stress suppresses not only helper T cell functions, but also the suppressor T cells. There are several lines of evidence to suggest that antibody response to at least some TI antigens are markedly influenced by suppressor T cells (11, 12).

In this article, we investigated the diverse effects of restraint stress on antibody responses in an in vitro culture system and tried to identify the stress-sensitive cells. The results of these experiments suggest that both functions of helper T cells and suppressor T cells are suppressed, but B cell activities in

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antibody responses to TI antigens are rather enhanced by restraint stress.

Materials and Methods

Animals: Female BALB/c mice (6 weeks of age) were purchased from Japan Charles River Breeding Laboratories (Tokyo, Japan) and were used during 10–14 weeks of age.

Immunological reagents and culture media:

The reagents used in the present experiments were obtained from the following sources: 2-mercaptoethanol (2-ME), Tokyo Kasei (Tokyo); α -methyl-D-mannoside, Sigma Chemical Company (St. Louis, MO); agar noble, Difco Laboratories (Detroit, MI). Fresh serum of guinea-pigs was used as the complement source. RPMI-1640 medium and Eagle's minimum essential medium (MEM) were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo). Fetal Calf Serum (FCS) (lot 26N4014) was obtained from Gibco Laboratories (Gland Island, NY). Culture medium was usually supplemented with penicillin G (100 unit/ml) and streptomycin (100 μ g/ml).

Antigens and mitogens: Sheep red blood cells (SRBC) stored in sterile Alserver's solution were obtained from Nishinippon Sheep Farm (Fukuyama, Japan). SRBC were washed twice with sterile saline before use. Dinitrophenylated (DNP)₃₆-Ficoll was prepared by coupling ϵ -DNP-L-Lysine (Sigma Chemical Co.) and Ficoll 400 (Pharmacia Fine Chemicals, Uppsala, Sweden) with cyanuric chloride (Wako Chemicals, Tokyo) as a cross-linker, according to the method of Haba and Hamaoka (13). Trinitrophenylated lipopolysaccharide (TNP-LPS) and Keyhole limpet hemocyanin (TNP-KLH) were prepared by reacting trinitrobenzene sulfonate (Tokyo Kasei, Tokyo) with LPS and KLH, respectively, by the procedure described by Rittenberg and Amkraut (14). LPS was the phenol-water extracts of *Escherichia coli* 055:B5 (Difco Laboratories, Detroit, MI). KLH was obtained from Calbiochem (San Diego, CA). Concanavalin A (Con A) was the product of E.Y. Laboratories (San Mateo, CA).

Culture of spleen cells for primary antibody response and assay of antibody-forming cells: Spleen cells (6×10^6) were cultured with

SRBC (2×10^6), DNP-Ficoll (20 ng/ml), TNP-LPS (0.5 μ g/ml) or TNP-KLH (0.1 μ g/ml) in 2 ml of RPMI-1640 medium supplemented with 10% FCS and 10^{-5} M 2-ME at 37°C in a humidified atmosphere of 5% CO₂ and 95% air for 4 days using 24-well multi-dish culture plates (Nunc, Kamstrup, Denmark). In the case of polyclonal antibody synthesis, the same number of cells were cultured in the presence of 50 μ g/ml LPS for 2 days. Antibody synthesis was assayed by enumerating the number of PFC by the method of Jerne and Nordin (15). Lightly conjugated TNP-SRBC were prepared as described by Rittenberg and Pratt (16) for detecting anti-TNP PFC. Polyclonal antibody production was assayed by using TNP-SRBC as the target.

DNA synthetic response: Using 96-well microculture plates (Nunc), spleen cells (2×10^5) were cultured in triplicate with Con A (2.5 μ g/ml) or LPS (25 μ g/ml) in 200 μ l of RPMI-1640 medium containing 5% FCS at 37°C for 48 hr in a humidified atmosphere of 5% CO₂ and 95% air. Cells were then radio-labeled for 18 hr with 0.5 μ Ci of [⁶⁻³H] thymidine ([³H] TdR) (20 mCi/mmol, Radiochemical Center Amersham, England). The cultures were harvested with an automatic cell harvester (Labo Science, Tokyo) and incorporated radioactivities were counted with a liquid scintillation counter.

Stressing procedure: Experimental mice were fixed in the 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 according to the literature (17). Usually, mice were restrained for 2 consecutive days. The control mice were allowed to remain in their home cages from which food and water were removed during the stress period of their counterparts.

Enrichment of B cells and T cells: Spleen cells (1×10^7) were incubated in 0.5 ml of MEM containing monoclonal anti-Thy-1.2 antibody (1:500, OIac, Blackthorn, England) for 30 min at 4°C. After centrifugation and washing once with MEM, the cells were resuspended in the original volume of MEM containing 1:10 volume of complement and incubated for 30 min at 37°C. T cell enrich-

ment was performed by passing spleen cells through a nylon wool column as described by Julius et al. (18).

Depletion of adherent cells: Spleen cells were depleted of adherent cells by passage over G-10 Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) by the method of Ly and Mishell (19).

Preparation of splenic adherent cells: Two hour glass-adherent cells (SAC) were prepared by the method described by Cowing et al. (20). All SAC populations were irradiated with 1300 R and used.

Preparation of Con A-induced suppressor cells: Spleen cells (6×10^6) were cultured for 48 hr with $10 \mu\text{g/ml}$ Con A or cultured without Con A as a control in 2 ml of RPMI-1640 medium supplemented with 10% FCS at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, according to the method of Rich and Pierce (21). These cells were then washed once with MEM containing 20 mg/ml α -methyl-D-mannoside and twice with MEM, and then they were resuspended in the culture medium.

Induction of SRBC-specific suppressor cells: Spleen cells (6×10^6) were cultured for 4 days with 2×10^6 SRBC or without SRBC as a control under the same conditions as described above according to the method of

Eardley and Gershon (22). These cells were washed two times with MEM and resuspended in the culture medium.

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

Restoration of the suppression of PFC response to SRBC in splenocytes from restrained mice by the addition of normal splenic T cells: The same number of spleen cells from control and restrained mice were cultured with SRBC for 4 days. As shown in Fig. 1, the PFC response of stressed cells was reduced to about 40% of that of the control cells. Addition of 6×10^5 normal splenic T cells into the stressed culture restored the response to nearly the control level. This data suggests that the suppression of PFC response in restrained mice is due to dysfunction of helper-T cells or activation of suppressor cells.

To ascertain the later possibility, keeping a constant cell density of 6×10^6 cells/well, spleen cells from control and restrained mice were mixed in various ratios and cultured with SRBC. As shown in Fig. 2, the observed anti-SRBC PFC response was very close to the

Fig. 1. Restoration of the suppressed PFC response to SRBC by the addition of normal T cells. Spleen cells (6×10^6) obtained from control and stressed mice were cultured with 2×10^6 SRBC in the presence or absence of the indicated number of splenic T cells (3 – 6×10^5) obtained from normal mice prepared utilizing a nylon wool column. Four days after culture, the number of anti-SRBC PFC was enumerated. The results represent the mean $\pm$ S.D. of triplicate cultures from a pool of four spleens. Significantly different from each control value: ** $P < 0.01$.

Fig. 2. Lack of suppressor activity in restrained mice. Control and stressed spleen cells were mixed in the ratios shown, keeping a constant density of 6×10^6 cells/culture. These cells were cultured with 2×10^6 SRBC for 4 days. The number of anti-SRBC PFC was enumerated. The results represent the mean $\pm$ S.D. of triplicate cultures from a pool of four spleens.

expected value. The expected values were calculated from the 100% values for the control cells. These results indicate that spleen cells from restrained mice contain few, if any, cells that can suppress the normal response.

Suppression of helper-T cell activities by restraint stress: The above data suggested that the suppression of antibody formation by restraint stress was due to dysfunction of helper T cells, but not to activation of suppressor cells. In order to clarify the influence of restraint stress on helper T cell functions, we measured helper T cell activity to respond against a hapten (TNP) conjugated to a carrier (KLH). Mice were restrained and then immunized intraperitoneally with 100 μ g KLH in alum. Two days after immunization, the splenocytes of these mice were cultured with 0.1 μ g/ml TNP-KLH, 20 ng/ml DNP-Ficoll or 0.5 μ g/ml TNP-LPS. Figure 3 shows that the PFC response to TNP conjugated with KLH in stressed cells was markedly suppressed as compared with the control cells. On the other hand, the responses to

the same TNP hapten conjugated with LPS was rather enhanced.

In order to confirm the suppression of helper T cells, we directly compared helper T cell activity with control and stressed mice. Splenic T cells from control and restrained mice were enriched by a nylon wool column and then reconstituted with normal B cells. As illustrated in Fig. 4, when 2×10^6 T cells from control mice were reconstituted, the anti-SRBC PFC response was almost recovered to the normal level at which normal whole spleen cells could generate. However, the same number of T cells from stressed mice had only one-half the recovery effect of control T cells, indicating the suppression of helper T cell functions in restrained mice. It was concluded that the suppression of the PFC response in restrained mice was due to dysfunction of helper T cells.

Inhibition of suppressor cell-induction by restraint stress: As shown in Fig. 2, restraint stress did not activate the suppressor cells. We examined the effects of restraint stress on the suppressor cell-induction. When 2×10^5

Fig. 3. Antibody response to TNP-carrier antigen in restrained mice after stress loading. BALB/c mice restrained for 2 days were intraperitoneally immunized with 100 μ g KLH with alum. Two days after immunization, spleen cells (6×10^6) of these mice were cultured with 0.1 μ g/ml TNP-KLH, 20 ng/ml DNP-Ficoll or 0.5 μ g/ml TNP-LPS. Anti-TNP PFC were enumerated 4 days thereafter. The results represent the mean $\pm$ S.D. of triplicate cultures from a pool of four spleens. Significantly different from each control value: ** $P < 0.01$.

Fig. 4. Activities of splenic T cells in restrained mice. Splenic T cells ($0.5-2 \times 10^6$) obtained from control and restrained mice by utilizing a nylon wool column were reconstituted with normal spleen cells depleted of T cells by the treatment with anti-Thy-1.2+complement and then cultured with 2×10^6 SRBC for 4 days. The results represent the mean $\pm$ S.D. of triplicate cultures from a pool of four spleens. Significantly different from each control value: ** $P < 0.01$.

Con A-induced cells from control mice were added to the normal culture, the PFC response to SRBC was suppressed to about 30% of the normal value. However, the same number of Con A-induced cells from stressed mice caused a response that was 75% of the normal level. These results are shown in Fig. 5. In the absence of Con A, cultured spleen cells either from control or stressed mice could not suppress the PFC response of normal cultures (data not shown).

Then, we determined the SRBC-specific suppressor cell activities in restrained mice. As shown in Fig. 6, 1×10^5 stressed cells educated with SRBC showed a lesser suppressive effect than control cells with regards

Fig. 5. Con A-induced suppressor cells in restrained mice. Spleen cells (6×10^6) obtained from control and restrained mice were precultured with $10 \mu\text{g/ml}$ Con A for 48 hr. These cells were washed with MEM and $0.5\text{--}2 \times 10^5$ of these cells were added to the normal fresh spleen cells (6×10^6) in the presence of 2×10^6 SRBC. Four days after culture, the number of anti-SRBC PFC were enumerated. The results represent the mean $\pm$ S.D. of triplicate cultures from a pool of four spleens. Significantly different from each control value: ** $P < 0.01$.

to the anti-SRBC PFC response. These results (Figs. 5 and 6) indicate that induction of suppressor cells in restrained mice is almost suppressed as well as helper-T cells.

Augmentation of antibody responses against T cell-independent antigens by restraint stress: In order to elucidate the influence of restraint stress on B cell activities, we investigated antibody responses to TI antigens in stressed spleen cells. Spleen cells from control and restrained mice were cultured with TI antigens, TNP-Ficoll or TNP-LPS for 4 days. As shown in Fig. 7A, the PFC responses of stressed cells were significantly augmented from 30 to 50% of those of control cells. In the absence of 2-ME in the culture medium, the same value of enhancement was observed (data not

Fig. 6. Induction of SRBC-specific suppressor cells in restrained mice. Spleen cells (6×10^6) obtained from control and restrained mice were precultured with 2×10^6 SRBC for 4 days. These cells were washed with MEM and $0.5\text{--}1 \times 10^5$ of these cells were added to the normal fresh spleen cells (6×10^6) in the presence of 2×10^6 SRBC. Four days after the second culture, the number of anti-SRBC PFC were enumerated. The results represent the mean $\pm$ S.D. of triplicate cultures from a pool of four spleens. Significantly different from each control value: ** $P < 0.01$.

Fig. 7. Augmentation of antibody responses against T cell-independent antigens by restraint stress. Spleen cells (6×10^6) obtained from control and restrained mice, which were not treated or depleted of T cells by the treatment of anti-Thy-1.2 + complement, were cultured with 2×10^6 SRBC, 20 ng/ml DNP-Ficoll or 0.5 μ g/ml TNP-LPS for 4 days. The number of anti-SRBC or anti-TNP PFC was enumerated. The results represent the mean $\pm$ S.D. of triplicate cultures from a pool of four spleens. Significantly different from each control value: * $P < 0.05$, ** $P < 0.01$.

shown). From these experiments, we got contradictory findings that antibody formations to TD antigens were suppressed, but those to TI antigens were rather enhanced in restrained mice.

We can propose two possible explanations for these findings. One is that B cells are directly activated to produce antibodies by stress loading. The other is that suppressor T cells are inactivated by the stress, resulting in the activation of B cells responding to TI antigens. Then, in order to exclude the influence of T cells, PFC responses against TI antigens were estimated using T cell-depleted spleen cells. Figure 7B shows that stressed cells depleted of T cells showed a similar augmentation of the PFC response as observed in whole spleen cells. These results indicate that B cells are directly activated by restraint stress.

Enhancement of polyclonal antibody response induced by LPS in stressed mice:

In order to ascertain the result obtained in Fig. 7, effect of restraint stress on polyclonal PFC response induced by LPS was examined. As illustrated in Fig. 8, stressed cells developed a PFC response that was about 2-fold that of control cells, indicating the promotion of B cell activities in restrained mice. It should be noted that stressed spleen cells used in Fig. 8 showed a lower PFC response against SRBC than that of control cells.

Comparison of B cell activities in control and restrained mice: The following experiment was carried out to clarify whether B cells responding to TD antigens such as SRBC were similarly activated by restraint stress as B cells responding to TI antigens. Normal splenic T cells were reconstituted with

B cells from splenocytes of control and with SRBC. As shown in Fig. 9, normal T restrained mice, respectively, and cultured cells reconstituted with stressed B cells

Fig. 8. Enhancement of polyclonal antibody response induced by LPS in stressed mice. Spleen cells (6×10^6) from control and restrained mice were cultured with 50 μ g/ml LPS for 2 days. The number of PFC was enumerated by using TNP-SRBC as the target. The results represent the mean $\pm$ S.D. of triplicate cultures from a pool of four spleens. Significantly different from each control value: ** $P < 0.01$.

Fig. 9. Comparison of B cell activities in control and restrained mice. T cell-depleted spleen cells (6×10^6) obtained from control and restrained mice were reconstituted with normal splenic T cells (0.5 – 2×10^6) prepared by utilizing a nylon wool column and cultured with 2×10^6 SRBC for 4 days. The number of anti-SRBC PFC in the culture was enumerated. The results represent the mean $\pm$ S.D. of triplicate cultures from a pool of four spleens.

Fig. 10. Comparison of activities of splenic adherent cells in control and restrained mice. Normal spleen cells were depleted of adherent cells by passage through G-10 columns and reconstituted with 2×10^6 SRBC for 4 days. The number of anti-SRBC PFC was enumerated. The results represent the mean $\pm$ S.D. of triplicate cultures from a pool of four spleens.

exhibited the same value of the PFC response as those recultured with control B cells. This result suggests that B cells responding to TD antigens were not influenced by restraint stress.

Comparison of activities of splenic adherent cells in control and restrained mice: We examined the activities of adherent cells from control and stressed splenocytes by comparing the ability for antigen presentation. Normal splenocytes were depleted of adherent cells by passage through G-10 columns and reconstituted with splenic adherent cells obtained from control or stressed mice. Figure 10 shows that $2\text{--}4 \times 10^5$ adherent cells from restrained mice have the same recovery effect as those from control mice in the anti-SRBC PFC response. This result indicates that restraint stress had few, if any, effects on the antigen presenting (AP) activity of splenic adherent cells.

Suppression of proliferative response to Con A, but not to LPS in restrained mice: To elucidate whether proliferation of splenic B cells are nonspecifically activated by restraint stress, effects of restraint stress on ^3H -TdR uptake into lymphocytes induced by mitogen were investigated. Figure 11 shows that Con A response was significantly suppressed in restrained mice, but LPS

response was not affected in these animals. With no mitogen, incorporation of ^3H -TdR into stressed cells were lower than that of control cells.

Discussion

Our present data clearly demonstrated that helper T cell functions are markedly suppressed in restrained mice because of the following three reasons: (1) The addition of normal splenic T cells completely recovered the suppressed PFC response of stressed cells. (2) The activity of T cells that interact with carrier antigen (KLH) to serve the helper function was suppressed by stress stimulus. (3) Splenic T cells from restrained mice could not restore the anti-SRBC PFC response in T cell-depleted spleen cells from normal mice.

On the other hand, data from several laboratories have demonstrated that Con A-activated mouse T cells, specifically Lyt-2,3^+ T cells, can suppress primary PFC responses to heterologous erythrocytes in normal cultured spleen cells (23–25). Moreover, Eardly and Gershon showed that spleen cells could be educated in vitro to induce T cell-dependent suppression of PFC response to SRBC (22). Feldman and Kontiainen reported that specific suppressor T cells can be

Fig. 11. Suppression of proliferative response to Con A, but not to LPS in restrained mice. Spleen cells (2×10^6) obtained from control and restrained mice were cultured with Con A ($2.5 \mu\text{g/ml}$) or LPS ($25 \mu\text{g/ml}$) for 48 hr. These cells were then radiolabeled for 18 hr with $0.5 \mu\text{Ci}$ of ^3H -TdR and harvested for counting incorporated radioactivities. The results represent the mean $\pm$ S.D. of triplicate cultures from a pool of four spleens. Significantly different from each control value: ** $P < 0.01$.

induced by incubating lymphoid cells *in vitro* with supra-immunogenic doses of antigen (26–28). By using these system, we found that induction of suppressor T cells was suppressed in restrained mice. Further, the proliferative response of stressed cells by Con A was reduced to 50% of the control. Feldman et al. have reported that suppressor cells are derived from an interaction of two T cell subpopulations: the suppressor cell amplifier and suppressor cell precursor (29). Although we demonstrated the inhibition of suppressor T cell-induction, we could not determine whether the suppression is due to dysfunction of the suppressor cell amplifier or the suppressor cell precursor. In conclusion, these results indicate that restraint stress suppresses the function of both helper T cells and suppressor T cells.

Our data showed that activity of splenic B cells is promoted in restrained mice, in contrast to the case of T cells. However, these enhancements were observed only when TI antigens were used. As illustrated in Fig. 9, the activity of B cells producing anti-

bodies to TD antigens from restrained mice was the same as those of B cells from control mice. From these data, we can propose a possibility. There may be at least two subpopulations of B cells that have a differential sensitivity to stress stimulus. That is to say, B cells secreting antibodies to TI antigens can be activated by restraint stress, but B cells responding to TD antigens are not affected. Some reports have been published about the existence of B cell subpopulations (7–10). Moreover, we found that in antibody response to TNP hapten, the response to TNP conjugated with KLH was markedly suppressed by restraint stress, but the response to the same TNP hapten conjugated with LPS was rather enhanced (Fig. 3). Experiments are in progress to clarify whether there are B cell subpopulations having different sensitivities to stress stimulus.

Adherent cells in spleens from stressed mice would not be affected by restraint stress. Moreover, the results from Figs. 1 and 7 indicate that the antigen presentation

activity of adherent cells was not affected by restraint stress. That is to say, the suppression of anti-SRBC PFC response was completely recovered by the addition of T cells in stress culture (Fig. 1). T cell-depleted spleen cells from restrained mice exhibited the same PFC responses to TD antigens as those from control mice (Fig. 7). In conclusion, we confirmed that restraint stress suppressed both functions of helper and suppressor T cells, but rather enhanced B cell activities in antibody responses to TI antigens.

Other investigators have suggested that a single stressor may differentially affect the host's immune response (30, 31). Our data are also consistent with these observations because restraint stress had a differential effect on immunocompetent cells, B cells, T cells and adherent cells.

The mechanism whereby stress may facilitate or suppress antibody response in mice is unknown. We found that the restraint stress used in the present study causes a two-fold increase in plasma corticosterone (T. Okimura et al., unpublished data). This elevation of glucocorticoids in stressed animals and the immunoregulatory actions of glucocorticoids would suggest that these hormones may be concerned with stress-induced alterations of antibody response (32–35). However, both suppressed and enhanced antibody response induced by the stress is difficult to explain only by an increase in serum glucocorticoids. This conclusion implies that other hormones or factors may also be responsible for the results observed in this study. Further studies as to hormonal mechanisms and other factors concerned with stress-induced alterations of antibody response are in progress.

Some correlations between stress and cancer and autoimmune diseases have been postulated, that is to say, effects of stress on induction and course of autoimmune diseases (36–38) and on the courses of growth of a variety of experimental tumors in animals (39–42). Restraint-induced suppression of T cell functions and augmentation of B cell activities, which were observed in our experiments, may be involved in the pathogenesis and course of cancer and/or autoimmune diseases. Therefore, it is important

to elucidate these correlations between stress and immune responses.

Acknowledgment: We wish to thank Prof. Dr. I. Yamamoto and Assistant Prof. Dr. H. Ohmori (Department of Immunochimistry, Okayama University) for helpful discussions.

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