Note

Immunoglobulin Secretions in the Mesenteric Lymph Node in Stressed Rats

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Summary  To study the effects of different types of stressors on intestinal immune function, the lymphocyte subsets and associated immunoglobulin production in stressed rats were observed. Physical (electric foot shock) or psychological (non foot-shock) stress respectively were induced using a communication box. Rats were exposed to stress for 2 h per day, and the treatment was maintained for 14 consecutive days. Lymphocytes were isolated from the mesenteric lymph node (MLN) and spleen using Lympholyte-Rat. There was no change the lymphocyte subsets in MLN or spleen in either group. Foot-shock stress increased immunoglobulin secretions in MLN lymphocytes. These results demonstrated that intestinal immune functions were adaptively regulated under conditions of moderate stress.

Key Words  physical stress, psychological stress, immunoglobulin, mesenteric lymph node (MLN)

Stress is associated with alterations in humoral and cellular immune functions in both human and laboratory animals. Physiological responses induced by stress involve activation of both the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system \textsuperscript{(1)}, with increments in plasma glucocorticoids and catecholamines \textsuperscript{(2)}. Many studies report that changes in the number and function of immunocytes in the thymus, spleen, or peripheral blood are attributable to stress \textsuperscript{(3, 4)}. In addition, stress can increase epithelial permeability and stimulate ion and water secretion and mucus release in the gastrointestinal tract. It can also influence immune cells and associated cytokine production. There appears to be a stress-activated pathway from the central nervous system to the mucosal immune cells \textsuperscript{(5)}. However, the mechanism by which stress induces immunomodulation is still not sufficiently understood.

The intestinal mucosa are continuously exposed to an immense load, consisting of antigens ingested with food, resident bacteria, invading viruses, etc. The epithelial layer lining the gut lumen possesses conflicting functions, playing a major role in the digestion and absorption of nutrients, and simultaneously constituting the most important barrier between the internal and external environments of an organism \textsuperscript{(6, 7)}. An increase in intestinal permeability in response to stress has been demonstrated in both animals and humans and in cases of both acute \textsuperscript{(8, 9)} and chronic stress \textsuperscript{(10)}. In early reports on stress-induced permeability changes (6, 8, 11), restraint stress obviously demonstrated barrier dysfunction (5, 12). As a result, an increase in intestinal permeability is followed by an increase in antigen invasion; and an increase of this nature probably influences intestinal immunity to a considerable extent. However, the influence of stress on intestinal immunity has not been clarified in detail thus far.

In this study, we have therefore examined the influence of stress on immunoglobulin secretion. In addition, we compared the effects of physical and psychological stressors on immune function. We attempted to determine whether different types of stressors might induce changes in the lymphocyte subsets and associated immunoglobulin production.

Physical and psychological stresses were induced using a communication box \textsuperscript{(13, 14)}. The communication box method is a useful model for investigating the physiological changes that occur under psychological stress, since it can induce experimental anxiety based on inraspecies emotional communication without generating direct physical stress. In this procedure, non-foot shocked rats were exposed to visual, olfactory, and auditory stimuli from foot-shocked rats. The experimental emotional responses of foot-shocked rats can induce an experimental anxiety in non-foot shocked rats \textsuperscript{(13, 14)}.

Materials and Methods

Animals. Male Sprague-Dawley rats of 6 wk of age were obtained from Kyudo (Saga, Japan). They were housed in individual cages for 2 wk, and randomly assigned to 3 experimental groups, namely control,
physical stress and psychological stress groups. The rats were housed in a temperature-controlled room (22–23°C) with a 12 h-light/12 h-dark cycle (light conditions from 8:00 to 20:00) and with free access to food (CE-2; CREA Japan) and water. At the end of the stress load period, blood was withdrawn from the abdominal vein under diethyl ether anesthesia and serum was harvested. This procedure was carried out in accordance with the Guidelines for Animal Experiments approved by the Prefectural University of Kumamoto.

Stress procedure. Physical and psychological stresses were induced using a communication box (Finecs (S) Pte, Ltd.). Rats in the physical stress group (FS group) were administered daily electric foot shocks for 14 d. These shocks were administered using an electric foot shock generator that produced 2 mA electric shocks of 10-s duration at intervals of 50 s for 2 h per day. Rats in the psychological stress group (PSY group) were prevented from receiving electric foot shocks by acrylic plates placed on an electric grid, which protected the rats from the shocks. Rats in the PSY group were exposed to emotional responses from the foot-shock rats.

In previous reports (2, 4, 13, 15), stress loads were employed for 1 d in acute stress response assessment. In the present experiment, by contrast, a stress load period of 6–14 d was employed, in order to assess chronic effects. In this study, the stress was employed for 14 d.

Preparation and cultivation of lymphocytes. Mesenteric lymph nodes (MLN) and spleen were removed immediately following etherization, minced in Roswell Park Memorial Institute (RPMI) 1640 (Gibco), and mashed on glass slides. Lymphocytes were isolated from these tissues using Lympholyte-Rat (Cedarlane) (16). The lymphocytes, 2.0×10^6 cells/mL, were cultured in an RPMI 1640 medium containing 10% fetal bovine serum (FBS) with or without 5 μg/mL concanavalin A (Con A), and were incubated at 37°C for 24 h in a 5% CO₂ atmosphere.

Lymphocyte population analysis. The cell surface expressions of CD3 (as a T cell marker), CD45RA (as a B cell marker), CD4, CD8, αβ TCR, and γδ TCR (as T cell subpopulation markers) were analyzed by flow cytometry (FACS Calibur HG flow cytometer; Becton Dickinson). Following the isolation of lymphocytes, cells were exposed to phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies. All antibody reactions were performed on ice for 30 min, and cells were washed 3 times with phosphate-buffered saline (PBS) following the antibody treatment. Samples were subjected to flow cytometry and a total of 10^4 cells were analyzed to determine the percentage of each positive lymphocyte type.

Measurement of immunoglobulin levels. Following 24-h culture of lymphocytes, immunoglobulin concentration in the cultured medium without Con A was determined using sandwich enzyme-linked immunosorbent assay (ELISA). Goat anti-rat IgA (Bethyl) and goat anti-rat IgG (Protos Immunoresearch) were employed to fix each immunoglobulin. These antibodies were diluted in a 50 mM carbonate-bicarbonate buffer, added to a 96-well plate and incubated for 1 h at 37°C. Subsequently, 4% Block Ace (Dainippon Sumitomo Pharma Co., Ltd.) was added and maintained at 4°C overnight. Samples were added to each well for 1 h at 37°C. Each well was next treated with a solution of either horseradish peroxidase (HRP)-conjugated goat anti-rat IgA (Bethyl) or HRP-conjugated goat anti-rat IgG (MP Biomedicals, Inc., Cappel) in order to detect the respective immunoglobulin. These were then incubated for 1 h at 37°C. These antibodies were diluted with 10% Block Ace. The plates were rinsed with PBS containing 0.05% Tween 20 between each step. Subsequently, a 10:9:1 mixture of 0.006% H₂O₂ in 0.2 M citrate buffer (pH 4.0), water, and 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Wako Pure Chemical Industries, Ltd.) was added. Finally, following the addition of 1.5% oxalic acid to arrest the reaction, absorbance at 415 nm was measured.

Measurement of cytokine levels. We measured the interferon (IFN)-γ and interleukin (IL)-4 levels in the Con A-stimulated lymphocytes supernatants. These were determined using commercial ELISA kits (BioSource International, Inc.).

Statistical analysis. Each result was expressed as a mean ± standard deviation (SD). All the data were compared between all groups, using the Tukey test at a 5% significance level.

Results and Discussion

The rats demonstrated changes in body weight and food intake at chronic stress load with a decreasing tendency. The body weight gain in the FS group was significantly lower than that in the control group (control, 9.21±1.18; FS, 5.53±1.64**; **p<0.01). In addition, there was a marked decrease in food intake due to stress as compared to the control group (control, 24.55±1.67; FS, 19.50±2.00**; **p<0.01). In the PSY group, the food intake rate decreased; however, no decrease in

Fig. 1. Effect of physical (FS) or psychological (PSY) stress on plasma corticosterone concentration. Rats were exposed to electric foot shock and non-foot shock stress using the communication box for 2 h per day. The values are expressed as the mean±SD of 9–15 rats per group. *p<0.05, compared with the control group.
Body weight gain was observed. Further, the increase in the fetal adrenal weight of the FS group was greater than in the control group (control, 14.81 ± 1.68; FS, 18.72 ± 3.45**; **p<0.01, compared with the control group). Therefore, it appears that physical stress induced HPA-axis activation. These results are in accordance with previous reports and confirm that altered HPA-axis activity is associated with the chronic physical stress regimen utilized in our investigation.

As previously reported (3, 17), stress exerted a profound effect on the cell number of lymphocyte subsets rather than on the proportion of lymphocyte subsets in the MLN or spleen lymphocytes. In this study, no significant changes in the abovementioned lymphocytes were observed in the groups (data not shown). It has been shown that changes in the proportion of lymphocyte subsets in the MLN or spleen do not occur under either physical or psychological stress conditions (3).

Stress-induced declines in the immune function have been thoroughly investigated (15, 18, 19). Some researchers have reported stressor-induced suppression of antibody production in rats or mice (20). In this study, no differences in the distribution pattern of lymphocyte subsets were observed; but we did observe stimulation of immunoglobulin production in physical stress-induced MLN lymphocytes (Fig. 2). By contrast, immunoglobulin secretions in the spleen lymphocytes did not demonstrate significant changes (data not shown) in the FS or PSY group. Further, compared with the control or PSY group, total plasma IgG levels demonstrated a significant increase in the FS group (Fig. 3). These results suggested that the significant increase in plasma IgG levels and in IgA levels of MLN lymphocytes may be induced by increased entry of antigens to be accompanied by gut hyperpermeability.

In the FS group there was significant suppression of the Th1-type humoral immune response in MLN lymphocytes...
Table 1. Effect of physical (FS) or psychological (PSY) stress on lymphocyte IFN-γ production. Rats were exposed to electric foot shock or non-foot shock stress using the communication box for 2 h per day.

|       | Control | FS     | PSY    |
|-------|---------|--------|--------|
| MLN lymphocytes (INF-γ, pg/mL) | 1.607.25±133.19 | 977.88±288.46 | 1.522.56±347.02 |
| Spleen lymphocytes (INF-γ, pg/mL) | 2.942.88±632.19 | 3.172.25±586.79 | 2.746.31±308.03 |

Values represent mean±SD (n=4). *p<0.05, compared with the control group. †p<0.05, compared with the FS group.

lymphocytes (Table 1). IL-4 production of MLN and spleen lymphocytes did not demonstrate a significant increase (data not shown). This result suggested that physical stress may affect the Th1/Th2 cytokine balance.

Although, in general, stress has been considered to be immunosuppressive, this study demonstrated that MLN lymphocytes functions were maintained and that IgA and IgG production against various antigens was increased. It is therefore suggested that these intestinal immune functions might be important for host defense under stress conditions. These results indicated that these intestinal immune functions were adaptively regulated under moderate stress conditions.

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