Tohoku J. Exp. Med., 2005, **206**, 203-212

Repeated Stress Suppresses Interferon-γ Production by Murine Intestinal Intraepithelial Lymphocytes

Xiumin Zhang, Mitsuharu Okutsu, Osamu Kanemi, Bahiru Gametchu[1] and Ryoichi Nagatomi

Department of Medicine and Science in Sports and Exercise, Tohoku University Graduate School of Medicine, Sendai, Japan, and
[1]Department of Pediatrics, Medical College of Wisconsin, Milwaukee, WI, USA

Zhang, X., Okutsu, M., Kanemi, O., Gametchu, B. and Nagatomi, R. *Repeated Stress Suppresses Interferon-γ Production by Murine Intestinal Intraepithelial Lymphocytes*. Tohoku J. Exp. Med., 2005, **206** (3), 203-212 ── Intestinal intraepithelial lymphocytes (IEL), one of the major effector components in the mucosal immune system, are phenotypically and functionally distinct from thymic and peripheral T cells. To investigate the effect of repeated stress on the number and function of IEL, we exposed male C3H/HeN mice to mild electric foot shock for 30 min/day for 5 consecutive days. Immediately after the final foot shock stress, the blood, spleen, thymus and small intestine of each of the mice were obtained. As a functional measure, we evaluated interferon (IFN)- γ production by IEL, since IFN-γ is a key immunomodulating cytokine in mucosal immune responses. Serum corticosterone level was elevated immediately after foot shock stress. There were no significant changes in the number of whole IEL and CD3$^+$ IEL subsets after the stress. In contrast, the stress led to a significant decrease in the total number of thymocytes, particularly the reduction in the number of CD4$^+$CD8$^+$ thymocytes. Thymocytes expressed the highest level of intracellular glucocorticoid receptor (GR), followed by splenocytes and IEL. The foot shock stress induced a marked suppression of IFN-γ production by IEL, when stimulated with immobilized anti-CD3 monoclonal antibody. Furthermore, corticosterone suppressed the IFN-γ production by cultured IEL, which was prevented by Mifepristone (RU486), a GR antagonist. In summary, repeated foot shock stress did not alter the numbers of IEL and CD3$^+$ IEL subsets, but suppressed IFN-γ production by IEL, which was probably mediated by the elevated corticosterone. We therefore propose that stress influences host defense by suppressing the production of IFN-γ in IEL. ──── stress; intestinal intraepithelial lymphocytes; interferon-γ; corticosterone

Intestinal intraepithelial lymphocytes (IEL), one of the major effector components in the mucosal immune system, have unique phenotypical and functional features compared with T cells from other lymphoid compartments (Matsuzaki et al. 1994; Mattapallil et al. 1998; Yoshikai 1999). IEL from the small intestine are composed of predominantly CD3$^+$, in which CD8$^+$ lymphocytes are more prevalent than CD4$^+$ (Lepage et al. 1998). In addition, most CD8$^+$ IEL express CD8

Received February 3, 2005; revision accepted for publication April 13, 2005.

Correspondence: Xiumin Zhang, Ph.D., Department of Medicine and Science in Sports and Exercise, Tohoku University Graduate School of Medicine, 2-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan.
e-mail: xmzhang@mail.tains.tohoku.ac.jp

$\alpha\alpha$ homodimers rather than the CD8$\alpha\beta$ heterodimers typically present on peripheral CD8 T cells (Guy-Grand et al. 1991; Eisenbraun et al. 2000). IEL from the murine small intestine comprise an approximately equal frequency of population bearing $\alpha\beta$TCR and $\gamma\delta$TCR.

IEL may produce various cytokines such as interferon (IFN)-γ, interleukin (IL)-5, IL-6, IL-10, transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α and keratinocyte growth factor (KGF) (Barrett et al. 1992; Boismenu and Havran 1994; Van Damme et al. 2001), regulate the immune responses to foreign antigens (Yamamoto et al. 1993; Culshaw et al. 1997) and modulate function of intestinal epithelial cells (IEC) (Madara and Stafford 1989; Kohyama et al. 1997; Taylor et al. 1997; Hoffman 2000).

IEC heavily express IFN-γ receptors (Valente et al. 1992), and respond well to IFN-γ. In vitro, IFN-γ alters the tight junction permeability between enterocytes (Madara and Stafford 1989; Adams et al. 1993), upregulates the expression of MHC class I and II antigens (Kohyama et al. 1997; Hoffman 2000), coinduces nitric oxide (NO) synthesis by IEC (Hoffman 2000), and enhances the expression of secretory component (SC) as a polymeric immunoglobulin receptor (pIgR) for dimeric IgA to form secretory IgA (S-IgA) (Sollid et al. 1987; Kvale et al. 1988; Youngman et al. 1994; Brandtzaeg et al. 1999; Nilsen et al. 1999). Moreover, IFN-γ producted by IEL plays an important role in resistance to infections from organisms such as *Listeria monocytogenes* and *Cryptosporidium* (Yamamoto et al. 1993; Culshaw et al. 1997). Altogether, IFN-γ production by IEL has an important role in both intestinal epithelial homeostasis and host defense.

Many studies have shown that stress induces changes in the number and function (especially IFN-γ production) of immune cells such as those in the thymus, spleen, lymph node or peripheral blood (Glaser et al. 1986; Sonnenfeld et al. 1992; Fukui et al. 1997; Iwakabe et al. 1998; Shimizu et al. 2000). Although the importance of IEL in the intestinal mucosal system is well established, the effect of stress on IEL has not been elucidated. The aim of this study is, therefore, to clarify the effect of stress on the number of IEL and IFN-γ production by IEL.

MATERIALS AND METHODS

Mice

Six-week-old male C3H/HeN mice were purchased from Japan SLC Inc. (Hamamatsu) and kept under specific-pathogen-free conditions in the Institute for Animal Experimentation, Tohoku University Graduate School of Medicine. The purchased mice were allowed to acclimate to the facility for at least one week before the experiment, and they were used at 7-9 weeks of age. Four or five mice were housed together per cage (30 × 25 × 17.5 cm) and were allowed free access to food and water. All experiments were performed according to the Guidelines and Regulations for Laboratory Animal Care of Tohoku University Graduate School of Medicine.

Protocol for foot shock stress

Foot shock stress was applied to the mice according to the method reported previously with some modification (Sonnenfeld et al. 1992). Direct electric current was applied to mice placed on steel bars in a limited plexiglas compartment (10 × 10 × 10 cm). Since stressors are considered to become clinically relevant especially when applied repeatedly, a foot shock pulse with a duration of 0.5 second was given every 5 seconds for 30 minutes (min) a day for five consecutive days. The foot shock voltage was adjusted to be as low as possible, just enough to elicit behavioral changes in the mice such as jumping or squeaking. The actual electric current measured serially

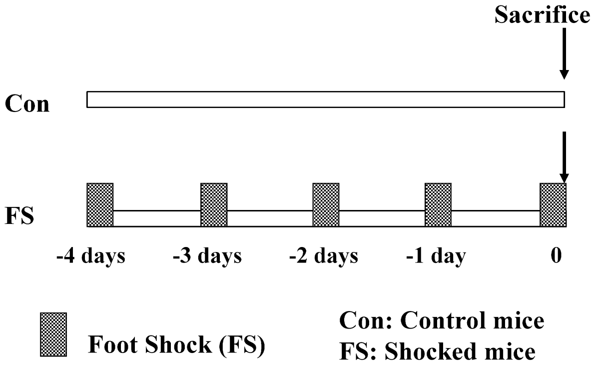

Fig. 1. Foot shock stress protocol. Mice were treated with 0.5 second electric foot shock every 5 seconds for 30 min every day for 5 consecutive days. Immediately after the final shock exposure, the shocked and control mice were sacrificed.

did not exceed 1 mA. The foot shock experiment was carried out in the morning between 9:00 and 10:00 a.m. Control mice of the same age were kept in their home cage throughout the foot shock procedure, where they could not perceive the reaction of foot-shocked mice during the foot shock procedure. Moreover, control mice were always kept in different cages from foot-shocked mice throughout the experiment. Immediately after the final shock exposure, shocked and control mice were sacrificed by decapitation. Blood sample, spleen, thymus and small intestine were obtained from each mouse. The protocol is summarized in Fig. 1.

Isolation of IEL

IEL were obtained from the small intestine according to the method reported previously with a minor modification (Fujihashi et al. 1993; Kohyama et al. 1997, 1999). In brief, the small intestine was excised from the sacrificed mouse and thoroughly washed with cold Ca^{2+}, Mg^{2+} free Hanks balanced salt solution (HBSS; GIBCO, Grand Island, NY, USA) supplemented with 10 mM N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES; Wako, Osaka), 25 mM sodium bicarbonate (Wako) and 2% heat-inactivated fetal bovine serum (FBS; Sigma, St. Louis, MO, USA). The mesenteric lymph nodes, connective tissue and Peyer's patches were carefully removed. The washed intestine was then opened longitudinally, and cut into short segments. The segments were transferred to a 50-ml conical tube containing 40 ml HBSS supplemented with 10 mM HEPES, 25 mM sodium bicarbonate, 10% FBS and 1mM dithioerythritol (Sigma), and horizontally shaken at 37°C for 45 min in a water bath. The cell suspension was filtered through a 70-μm cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and then passed through a nylon fiber column. Cells contained in the filtrate were suspended in 30% Percoll solution (Pharmacia, Uppsala, Sweden) and centrifuged at room temperature for 20 min at 600 g. Pelleted cells were then resuspended in 40% Percoll solution and carefully layered over 75% Percoll solution, and finally centrifuged at room temperature for 20 min at 600 g. IEL were recovered from the interface of the 40% and 75% Percoll solutions. The collected cells were washed and resuspended in culture medium (RPMI 1640 [Sigma] supplemented with 10 mM HEPES, 100 unit/ml penicillin [Sigma], 100 μg/ml streptomycin [Sigma], 2 mM L-glutamine, and 10% FBS). Viable IEL were enumerated by trypan blue exclusion counting on a hemocytometer.

Preparation of thymocytes and splenocytes

Thymocytes and splenocytes were prepared according to the method reported previously with a minor modification (Sonnenfeld et al. 1992; Concordet and Ferry 1993). The spleen and thymus were removed from sacrificed mice and passed through a sterile wire mesh to remove tissue debris. The cell suspension was then centrifuged, and the cell pellet was mixed with 0.83% NH_4Cl Tris buffer to lyse the red blood cells. Cells were then washed three times and resuspended in the culture medium described above. Viable cells were enumerated by trypan blue exclusion counting on a hemocytometer.

Antibodies

Monoclonal antibodies (mAb) used in this study were purchased from BD Biosciences (San Jose, CA, USA) with the exception of mouse anti-CD4-FITC (JEP402), which was purchased from GIBCO, and mouse anti-glucocorticoid receptor (GR)-FITC (BUGR-2), which was produced as previously reported (Gametchu and Harrison 1984). Purified mouse anti-CD3 mAb (145-2C11) were used to activate T cells. Mouse anti-CD3-PerCP (145-2C11), anti-$\alpha\beta$TCR-FITC (H57-597), anti-$\gamma\delta$TCR-PE (GL3), anti-CD4-FITC (JEP402), anti-CD8α-PE and anti-CD8β-FITC (53-5.8) mAb were used for surface staining. Anti-GR-FITC (BUGR-2) mAb were used for surface or intracellular staining.

Flow cytometric analysis of cell surface antigens

Cells were incubated with mouse serum (Sigma) for 30 min at 4°C to block nonspecific antibody binding via IgG Fc receptors. After staining with the designated combination of mAb for 30 min at 4°C followed by washing twice with phosphate buffered saline (PBS), cells were suspended in PBS containing 1% paraformaldehyde (Wako) and analyzed on a FACScan flow cytometer using CELLQuest software (BD Biosciences). Lymphocytes were gated by size and granule density based on forward and side scatter (Van Houten et al. 1997).

Flow cytometric analysis of intracellular and membrane GR expression in IEL, thymocytes and splenocytes

Cells were incubated with mouse serum to block nonspecific antibody binding via the IgG Fc receptors. To determine intracellular GR expression, after the surface markers were stained with anti-CD3-PerCP, the cells were washed twice, fixed and permeabilized with

Permeabilizing Solution (BD Biosciences). The permeabilized cells were stained with anti-GR (BuGR-2)-FITC mAb. To determine membrane GR expression, after the IgG Fc receptors were blocked as explained above, the cell surface markers were stained with anti-CD3-PerCP and anti-GR (BuGR-2)-FITC mAb. Stained cells were washed twice, suspended in PBS containing 1% paraformaldehyde, and analyzed by flow cytometry.

Cell culture procedure and measurement of IFN-γ in culture supernatant

A flat-bottom 96-well microtiter plate was coated with 100 μl purified mouse anti-CD3 mAb (10 μg/ml) overnight at 4°C. The remaining liquid phase was aspirated and washed twice with PBS, then used to activate IEL.

Freshly isolated IEL were cultured in a 96-well flat bottom anti-CD3-coated plate in a total volume of 200 μl in culture medium at 1×10^5 IEL/well for 48 h at 37°C in a humidified 5% CO_2 incubator. Cells were also incubated in a non-coated well as a control. Culture supernatants were harvested after 48 h of incubation, clarified by centrifugation and stored at –20°C until assayed for cytokine. Levels of IFN-γ in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA), using a commercially available kit for mouse IFN-γ immunoassay (R & D systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Effect of corticosterone on IFN-γ production by IEL stimulated with immobilized anti-CD3 mAb in vitro

Freshly isolated IEL were cultured in a 96-well flat bottom plate in a total volume of 200 μl in culture medium at 1×10^5 IEL/well at 37°C in a humidified 5% CO_2 incubator. Cells were stimulated with immobilized anti-CD3 mAb in the presence or absence of 10^{-6} M corticosterone (COR) (Sigma). To examine whether COR affects IFN-γ production is mediated through GR, IEL were incubated in culture medium containing 10^{-6} M COR with or without 10^{-6} M Mifepristone (RU486) (Sigma), a GR antagonist. Culture supernatants were harvested after 48-h incubation, clarified by centrifugation and stored at –20°C until assayed for IFN-γ.

Measurement of serum COR

Serum COR was determined by radioimmunoassay (RIA) using the COR-^{3}H RIA Kit (ICN Biomedicals, Inc., Costa Mesa, CA, USA), according to the manufacturer's instructions.

Statistical analysis

Data were expressed as means (S.E.). We examined statistical differences using Student's *t*-test or one-way analysis of variance (ANOVA). Post hoc analysis was carried out using Fisher's protected least significant difference (PLSD). All statistical analyses were made with StatView software (SAS Institute Inc., Cary, NC, USA). $P < 0.05$ was regarded as statistically significant.

RESULTS

Elevation of serum COR level after repeated foot shock stress

Foot shock stress induced a significant increase in the serum COR level (control mice: 26.51 (3.26) ng/ml versus shocked mice: 221 (15.72) ng/ml, $n = 8$, $p < 0.001$ by student's *t*-test).

Effect of repeated foot shock stress on the total number of IEL, thymocytes and splenocytes

As shown in Fig. 2, foot shock stress led to a significant decrease in the total number of thymocytes ($p < 0.05$). On the other hand, no effect of foot shock stress was observed in the total number of IEL and splenocytes.

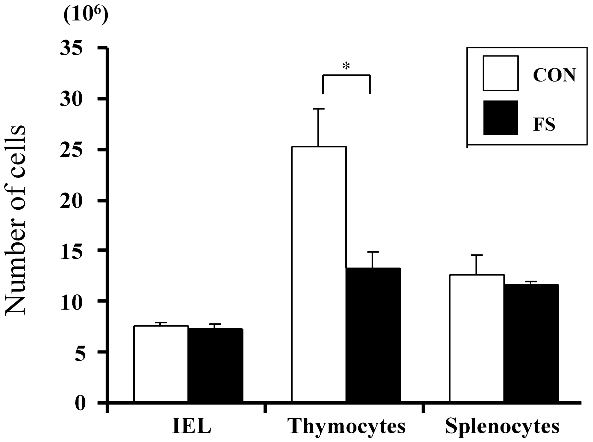

Fig. 2. Differential effects of foot shock stress on the number of IEL, thymocytes and splenocytes. Results are shown as the means (S.E.) of 6 separate experiments. For each experiment one mouse was assigned to each condition, and the same experiment was repeated 6 times. $^*p < 0.05$ (Student's *t*-test).

Effect of repeated foot shock stress on the number of T cell subsets in IEL, thymocytes and splenocytes

Foot shock stress induced significant reduction only in the number of CD4$^+$CD8$^+$ thymocytes ($p < 0.05$). The number of CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ thymocytes, however, showed a tendency to decrease, but not statistically significant (Fig. 3A). There were no significant changes in the number of CD4$^+$CD8$^-$, CD4$^-$CD8$^+$ and CD4$^+$CD8$^+$ IEL subsets after foot shock stress (Fig. 3B). In addition no splenocytes expressed CD4$^+$CD8$^+$, and foot shock stress did not induce any significant changes in the number in CD3$^+$ splenocyte subsets including CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ (Fig. 3C). Foot shock stress also induced no change in the number of $\alpha\beta$TCR$^+$ and $\gamma\delta$TCR$^+$ subsets of IEL and of splenocytes (data not shown).

CD8$^+$ IEL expressed either homodimeric CD8$\alpha\alpha$ (approximately 70%) or heterodimeric CD8$\alpha\beta$ (approximately 30%) molecules, and foot shock stress did not induce any significant changes in these two subsets although the number of CD8$\alpha\beta^+$ IEL showed a tendency toward reduction (Fig. 4).

Intracellular and membrane GR expression in IEL, thymocytes and splenocytes

There was a marked difference in the mean fluorescence intensity (MFI) of intracellular GR expression among thymocytes, splenocytes and IEL. Thymocytes had the highest level of intracellular GR expression, followed by splenocytes and IEL ($p < 0.001$) (Fig. 5). Thymocytes, splenocytes and IEL did not virtually express membrane GR (data not shown).

IFN-γ production by immobilized anti-CD3-stimulated IEL was suppressed after foot shock stress

IFN-γ production by IEL in 48-h culture supernatant was significantly reduced after foot shock stress (control mice: 2621.31 (366.5) pg/ml versus shocked mice: 1176.39 (286.45) pg/ml, $n = 4$, $p < 0.05$ by student's t-test). IFN-γ could not be detected in the 48-h culture supernatant when the cells had not been stimulated.

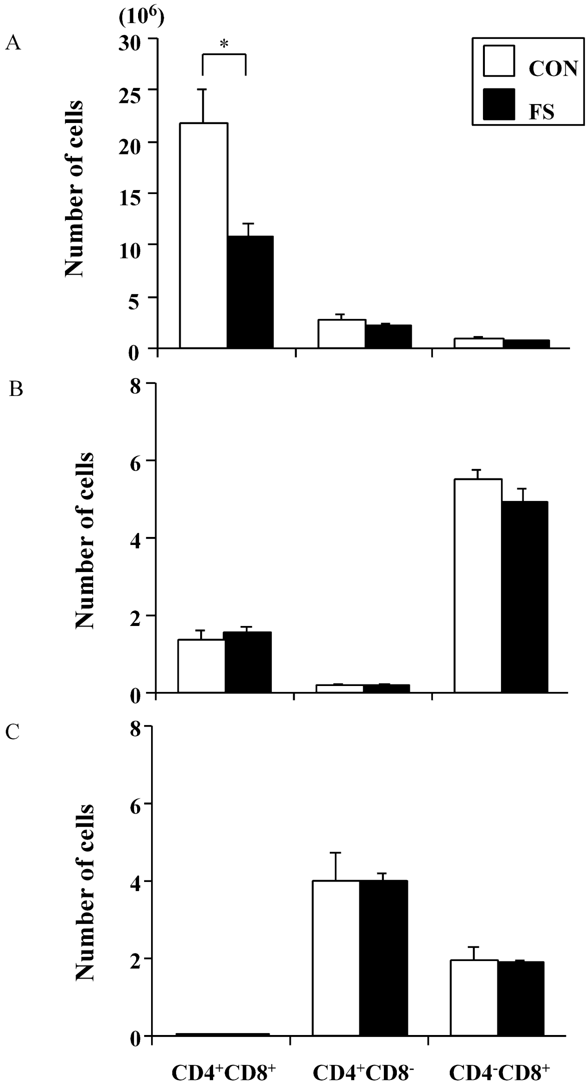

Fig. 3. Effect of foot shock stress on the number of CD4$^+$CD8$^+$, CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ subsets in thymocyte (A), IEL (B) and splenocyte (C). Thymocytes, IEL and splenocytes were stained with PerCP anti-CD3, FITC anti-CD4 and PE anti-CD8α mAb, and analyzed by flow cytometry for the expression of these lymphocyte subsets. The absolute number of each subset was obtained by multiplying the percentage of cells by the total number of cells in the respective populations. Results are shown as the means (S.E.) of 6 separate experiments. For each experiment one mouse was assigned to each condition, and the same experiment was repeated 6 times. $^*p < 0.05$ (Student's t-test).

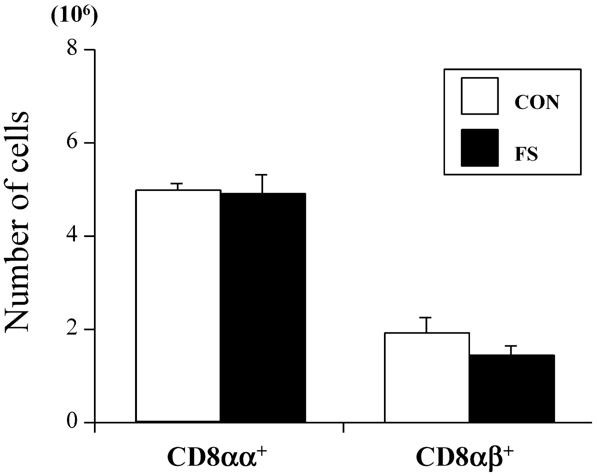

Fig. 4. Effect of foot shock stress on the number of CD8$\alpha\alpha^+$ and CD8$\alpha\beta^+$ IEL subsets. IEL were stained with PerCP anti-CD3, FITC anti-CD8β and PE anti-CD8α mAb, and analyzed by flow cytometry for the expression of CD8$\alpha\alpha^+$ and CD8$\alpha\beta^+$ IEL subsets in the gated CD3$^+$ IEL. The absolute number of each subset was obtained by multiplying the percentage of cells by the total number of cells in the respective populations. CD8$\alpha^+\beta^-$ IEL were considered to be CD8$\alpha\alpha^+$ IEL. Results are shown as the means (S.E.) of 6 separate experiments. For each experiment one mouse was assigned to each condition, and the same experiment was repeated 6 times.

COR suppressed IFN-γ production by IEL through GR in vitro

IFN-γ production by IEL was significantly reduced by COR ($p < 0.05$). RU486 alone did not alter IFN-γ production by IEL. However, RU486 effectively prevented the COR-induced suppression of IFN-γ production by IEL ($p < 0.05$) (Fig. 6), indicating that the suppression was mediated through GR.

DISCUSSION

This study first examined the effect of repeated foot shock stress on the number of IEL and IEL subsets in comparison with splenocytes and thymocytes, as well as the IFN-γ production by IEL. Because foot shock induced a marked increase in serum COR, the repeated foot shock stress employed in this experiment markedly re-

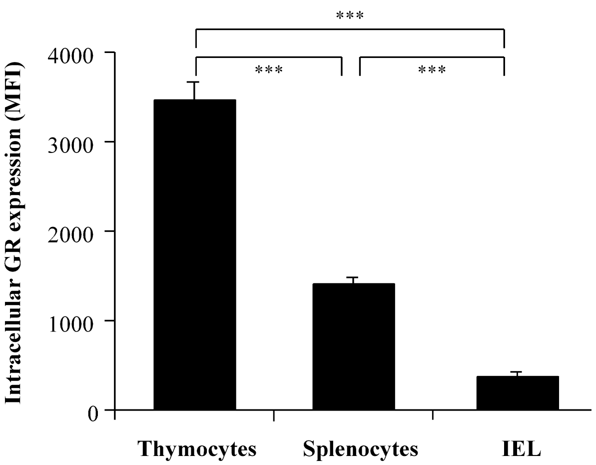

Fig. 5. Intracellular GR expression in thymocytes, splenocytes and IEL. To determine intracellular GR expression, after staining of the surface marker with anti-CD3-PerCP mAb, cells were fixed and permeabilized. The permeabilized cells were stained with anti-GR-FITC mAb. Stained cells were analyzed by flow cytometry. Data are shown as the means (S.E.) of 4 separate experiments. $^{***}p < 0.001$ (ANOVA with Fisher's PLSD post hoc test).

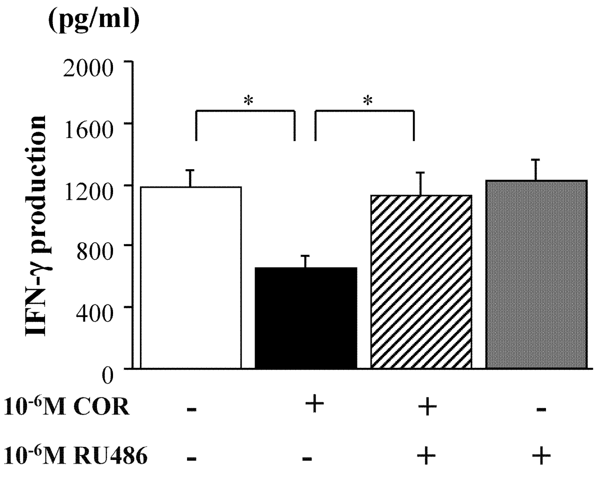

Fig. 6. RU486 prevented the COR-induced suppression of IFN-γ production in cultured IEL. IEL were stimulated with immobilized anti-CD3 mAb in the presence or absence of 10^{-6} M COR ± 10^{-6} M RU486 for 48 h. Supernatants were harvested and assayed for IFN-γ by ELISA. Results are shown as the means (S.E.) of 4 mice. $^{*}p < 0.05$ (ANOVA with Fisher's PLSD post hoc test).

duced the number of thymocytes, particularly $CD4^+CD8^+$ double positive thymocytes, most likely because of GR-mediated apoptosis under stress as reported by several investigators (Concordet and Ferry 1993; Murosaki et al. 1997). Interestingly, we could not demonstrate any decrease of IEL and splenocytes either in cell numbers or proportions of each subset.

Our flow cytometric analysis shows that thymocytes have the highest level of intracellular GR expression among thymocytes, splenocytes and IEL. This result is similar to that from the cytosolic GR binding study on immune tissues by Miller et al. (1998), who showed that the thymus exhibits three to four times higher levels of GR protein than the spleen. Van Houten et al. (1997) showed that IEL express the highest levels of anti-apoptotic protein Bcl-2, splenocytes express an intermediate intensity and thymocytes have the lowest level of Bcl-2 expression. Thus, IEL and splenocytes may have lower susceptibility to glucocorticoid-induced apoptosis as compared to thymocytes. The apparent difference in the susceptibility to stress among IEL, splenocytes and thymocytes may partly be resulting from the difference in the level of intracellular GR and anti-apoptotic protein Bcl-2 expression among lymphocyte populations.

Our data also demonstrated for the first time that susceptibility to foot shock stress is different between $CD4^+CD8^+$ IEL and $CD4^+CD8^+$ thymocytes. The reduction in the number of thymocytes after foot shock stress was because of the loss of $CD4^+CD8^+$ T cells, which are regarded as immature T cells (Reimann and Rudolphi 1995; Van Houten et al. 1997). Immature T cells have the highest susceptibility to glucocorticoid treatment leading to apoptosis (Wyllie 1980; Compton and Cidlowski 1986; Van Houten et al. 1997). In contrast, IEL expressing the same set of CD4 and CD8 surface proteins as on thymocytes, $CD4^+CD8^+$ IEL, were unaffected, suggesting that $CD4^+CD8^+$ IEL were resistant to stress-induced reduction.

In mice, in contrast to $CD8^+$ thymic T cells which almost exclusively express the heterodimeric $CD8\alpha\beta$ coreceptor, the $CD8^+$ IEL segregate into two populations, expressing either a homodimeric $CD8\alpha\alpha$ or a heterodimeric $CD8\alpha\beta$ molecule (Emoto et al. 1996). Experiments using athymic and nude mice showed that $CD8\alpha\alpha$ T cells in the intestinal epithelium were thymus-independent, whereas $CD8\alpha\beta$ T cells were thymus-dependent (Jarry et al. 1990; Guy-Grand et al. 1991; Rocha et al. 1991; Rocha et al. 1992; Poussier and Julius 1994). All $CD4^+CD8^+$ IEL bear $CD8\alpha\alpha$ homodimer chains, and are thus classified as extrathymic origin (Lefrancois 1991; Yamada et al. 1999). Therefore, $CD4^+CD8^+$ IEL are a distinct population from thymic $CD4^+CD8^+$ T cells. Van Houten et al showed that the treatment of mice with dexamethasone induced a marked reduction of $CD4^+CD8^+$ thymocytes, whereas $CD4^+CD8^+$ IEL were unaffected (Van Houten et al. 1997). This finding is apparently in accordance with our result. $CD4^+CD8^+$ IEL are considered to be a mature T cell population bearing relatively higher expression of CD3 and TCR $\alpha\beta$, as compared to the scant expression of the CD3-TCR complex on thymic $CD4^+CD8^+$ T cells (Lefrancois 1991; Fujihashi et al. 1993).

Although in this study repeated foot shock stress did not affect the number of IEL, a more intense acute stressor affected IEL. Murosaki et al. (1997) showed that 15 h water immersion stress led to a significant reduction in the number of IEL. Among $CD8^+$ IEL, $CD8\alpha\alpha^+$ IEL seemed to be relatively resistant to stress-induced reduction (Murosaki et al. 1997). We neither observed any significant change in the number of IEL nor in the proportion of $CD8\alpha\alpha^+$ or $CD8\alpha\beta^+$ IEL after repeated foot shock stress (although the number of $CD8\alpha\beta^+$ IEL tended to decrease). It is possible that the mild, short duration foot shock stress in our study was not as intense as 15 h water immersion stress to affect IEL number and phenotypes.

Although there was no significant difference in COR levels after 15 h water immersion stress and repeated foot shock stress in this study (10-fold and 9-fold of baseline, respectively), decrease in IEL number was induced only by 15 h water immersion stress but not by repeated foot shock stress. Even though GR level of IEL is relatively low in comparison with thymocytes and splenocytes, IEL are shown to be lost by apoptosis mediated by glucocorticoid (Murosaki et al. 1997). There are two possibilities to explain the difference. First, the duration of exposure to COR could be shorter in the foot shock paradigm. Secondly, repeated elevation of COR in repeated foot shock stress might have induced anti-apoptotic pathway. If the latter is true, elevated COR after repeated stress could suppress IFN-γ production without the loss of IEL by apoptosis. We will further test this hypothesis in our following studies.

Our major finding in this study was that repeated foot shock stress induced marked reduction of IFN-γ production by IEL in vitro, although no changes were observed in the number of IEL. Many previous studies have suggested that stress can affect cytokine production, especially IFN-γ (Sonnenfeld et al. 1992; Iwakabe et al. 1998; Moynihan et al. 1998; Zhang et al. 1998). These studies have mainly focused on the spleen, lymph nodes or peripheral blood lymphocytes, but not on IEL. In this study, it remains unclear what subsets of IELs may be affected by the stress to induce the suppression of IFN-γ production. We will therefore elucidate it using flow cytometry analysis of intracellular cytokine in our future studies.

We also tried to demonstrate the mechanism by which the IFN-γ production by IEL was suppressed. We confirmed that COR effectively suppresses the IFN-γ production by IEL through GR in vitro in a similar concentration observed after foot shock stress. Therefore elevated level of COR following the foot shock stress may be responsible for the reduction in the potential of IEL to produce IFN-γ upon stimuli in vitro.

Although not much is known about the role of IFN-γ production by IEL in vivo, in vitro experiments or murine models of infection suggested that IFN-γ production by IEL may play an important role in the functional regulation of IEC and in the host defense (Madara and Stafford 1989; Yamamoto et al. 1993; Culshaw et al. 1997; Kohyama et al. 1997; Taylor et al. 1997; Hoffman 2000), for example, by enhancing SC expression by IEC in the mucosal immune system (Sollid et al. 1987; Kvale et al. 1988; Youngman et al. 1994; Nilsen et al. 1999). It is possible that stress-induced suppression of IFN-γ production by IEL may affect the homeostasis of the intestinal epithelial barrier by suppressing S-IgA transport, and may cause a defective resistance to infection. Examination of serum and fecal IgA may help us to elucidate the contribution of IEL in vivo during the stress. Further studies are necessary to examine these possibilities.

In summary, we have demonstrated that thymocytes are more sensitive to stress-induced reduction than are IEL and splenocytes. This finding might be related to thymocytes having a higher level of GR and a lower level of bcl-2. Furthermore, our data first confirmed that CD4$^+$CD8$^+$ IEL are more resistant to stress than CD4$^+$CD8$^+$ thymocytes, which might be related to their different developmental pathways and stages. Repeated foot shock stress did not alter the numbers of IEL and CD3$^+$IEL subsets, but it suppressed the IFN-γ production by IEL, which may be related to the elevated corticosterone.

Acknowledgments

We thank Professor Hiroshi Nagura (Division of Athletics and Nutrition, Sendai College, Japan) for helpful scientific discussions and critical reading of the manuscript.

This work was supported by the Gonryo Medical Foundation.

References

Adams, R.B., Planchon, S.M. & Roche, J.K. (1993) IFN-γ modulation of epithelial barrier function. Time course, reversibility, and site of cytokine binding. *J. Immunol.*, **150**, 2356-2363.

Barrett, T.A., Gajewski, T.F., Danielpour, D., Chang, E.B., Beagley, K.W. & Bluestone, J.A. (1992) Differential function of intestinal intraepithelial lymphocyte subsets. *J. Immunol.*, **149**, 1124-1130.

Boismenu, R. & Havran, W.L. (1994) Modulation of epithelial cell growth by intraepithelial $\gamma\delta$ T cells. *Science*, **266**, 1253-1255.

Brandtzaeg, P., Baekkevold, E.S., Farstad, I.N., Jahnsen, F.L., Johansen, F.E., Nilsen, E.M. & Yamanaka, T. (1999) Regional specialization in the mucosal immune system: what happens in the microcompartments? *Immunol. Today*, **20**, 141-151.

Compton, M.M. & Cidlowski, J.A. (1986) Rapid in vivo effects of glucocorticoids on the integrity of rat lymphocyte genomic deoxyribonucleic acid. *Endocrinology*, **118**, 38-45.

Concordet, J.P. & Ferry, A. (1993) Physiological programmed cell death in thymocytes is induced by physical stress (exercise). *Am. J. Physiol.*, **265**, C626-629.

Culshaw, R.J., Bancroft, G.J. & McDonald, V. (1997) Gut intraepithelial lymphocytes induce immunity against Cryptosporidium infection through a mechanism involving gamma interferon production. *Infect. Immun.*, **65**, 3074-3079.

Eisenbraun, M.D., Mosley, R.L., Teitelbaum, D.H. & Miller, R.A. (2000) Altered development of intestinal intraepithelial lymphocytes in P-glycoprotein-deficient mice. *Dev. Comp. Immunol.*, **24**, 783-795.

Emoto, M., Neuhaus, O., Emoto, Y. & Kaufmann, S.H. (1996) Influence of β_2 -microglobulin expression on gamma interferon secretion and target cell lysis by intraepithelial lymphocytes during intestinal Listeria monocytogenes infection. *Infect. Immun.*, **64**, 569-575.

Fujihashi, K., Yamamoto, M., McGhee, J.R. & Kiyono, H. (1993) $\alpha\beta$ T cell receptor-positive intraepithelial lymphocytes with CD4$^+$, CD8$^-$ and CD4$^+$, CD8$^+$ phenotypes from orally immunized mice provide Th2-like function for B cell responses. *J. Immunol.*, **151**, 6681-6691.

Fukui, Y., Sudo, N., Yu, X.N., Nukina, H., Sogawa, H. & Kubo, C. (1997) The restraint stress-induced reduction in lymphocyte cell number in lymphoid organs correlates with the suppression of in vivo antibody production. *J. Neuroimmunol.*, **79**, 211-217.

Gametchu, B. & Harrison, R.W. (1984) Characterization of a monoclonal antibody to the rat liver glucocorticoid receptor. *Endocrinology*, **114**, 274-279.

Glaser, R., Rice, J., Speicher, C.E., Stout, J.C. & Kiecolt-Glaser, J.K. (1986) Stress depresses interferon production by leukocytes concomitant with a decrease in natural killer cell activity. *Behav. Neurosci.*, **100**, 675-678.

Guy-Grand, D., Cerf-Bensussan, N., Malissen, B., Malassis-Seris, M., Briottet, C. & Vassalli, P. (1991) Two gut intraepithelial CD8$^+$ lymphocyte populations with different T cell receptors: a role for the gut epithelium in T cell differentiation. *J. Exp. Med.*, **173**, 471-481.

Hoffman, R.A. (2000) Intraepithelial lymphocytes coinduce nitric oxide synthase in intestinal epithelial cells. Am. J. Physiol. Gastrointest. *Liver Physiol.*, **278**, G886-G894.

Iwakabe, K., Shimada, M., Ohta, A., Yahata, T., Ohmi, Y., Habu, S. & Nishimura, T. (1998) The restraint stress drives a shift in Th1/Th2 balance toward Th2-dominant immunity in mice. *Immunol. Lett.*, **62**, 39-43.

Jarry, A., Cerf-Bensussan, N., Brousse, N., Selz, F. & Guy-Grand, D. (1990) Subsets of CD3$^+$ (T cell receptor α/β or γ/δ) and CD3$^-$ lymphocytes isolated from normal human gut epithelium display phenotypical features different from their counterparts in peripheral blood. *Eur. J. Immunol.*, **20**, 1097-1103.

Kohyama, M., Hachimura, S., Nanno, M., Ishikawa, H. & Kaminogawa, S. (1997) Analysis of cytokine producing activity of intestinal intraepithelial T cells from TCR β-chain and δ-chain mutant mice. *Microbiol. Immunol.*, **41**, 353-359.

Kohyama, M., Nanno, M., Kawaguchi-Miyashita, M., Shimada, S., Watanabe, M., Hibi, T., Kaminogawa, S. & Ishikawa, H. (1999) Cytolytic and IFN-γ-producing activities of $\gamma\delta$ T cells in the mouse intestinal epithelium are T cell receptor-β-chain dependent. *Proc. Natl. Acad. Sci. USA*, **96**, 7451-7455.

Kvale, D., Brandtzaeg, P. & Lovhaug, D. (1988) Up-regulation of the expression of secretory component and HLA molecules in a human colonic cell line by tumour necrosis factor-α and gamma interferon. *Scand. J. Immunol.*, **28**, 351-357.

Lefrancois, L. (1991) Phenotypic complexity of intraepithelial lymphocytes of the small intestine. *J. Immunol.*, **147**, 1746-1751.

Lepage, A.C., Buzoni-Gatel, D., Bout, D.T. & Kasper, L.H. (1998) Gut-derived intraepithelial lymphocytes induce long term immunity against Toxoplasma gondii. *J. Immunol.*, **161**, 4902-4908.

Madara, J.L. & Stafford, J. (1989) Interferon-γ directly affects barrier function of cultured intestinal epithelial monolayers. *J. Clin. Invest.*, **83**, 724-727.

Matsuzaki, G., Lin, T. & Nomoto, K. (1994) Differentiation and function of intestinal intraepithelial lymphocytes. *Int. Rev. Immunol.*, **11**, 47-60.

Mattapallil, J.J., Smit-McBride, Z., McChesney, M. & Dandekar, S. (1998) Intestinal intraepithelial lymphocytes are primed for gamma interferon and MIP-1β expression and display antiviral cytotoxic activity despite severe CD4$^+$ T-cell depletion in primary simian immunodeficiency virus infection. *J. Virol.*, **72**, 6421-6429.

Miller, A.H., Spencer, R.L., Pearce, B.D., Pisell, T.L., Azrieli, Y., Tanapat, P., Moday, H., Rhee, R. & McEwen, B.S. (1998) Glucocorticoid receptors are differentially expressed in the cells and tissues of the immune system. *Cell. Immunol.*, **186**, 45-54.

Moynihan, J.A., Callahan, T.A., Kelley, S.P. & Campbell, L.M. (1998) Adrenal hormone modulation of type 1 and type 2 cytokine production by spleen cells: dexamethasone and dehydroepiandrosterone suppress interleukin-2, interleukin-4, and interferon-γ production in vitro. *Cell. Immunol.*, **184**, 58-64.

Murosaki, S., Inagaki-Ohara, K., Kusaka, H., Ikeda, H. & Yoshikai, Y. (1997) Apoptosis of intestinal intraepithelial lymphocytes induced by exogenous and endogenous glucocorticoids. *Microbiol. Immunol.*, **41**, 139-148.

Nilsen, E.M., Johansen, F.E., Kvale, D., Krajci, P. & Brandtzaeg, P. (1999) Different regulatory pathways employed in cytokine-enhanced expression of secretory component and epithelial HLA class I genes. *Eur. J. Immunol.*, **29**, 168-179.

Poussier, P. & Julius, M. (1994) Thymus independent T cell development and selection in the intestinal epithelium. *Annu. Rev. Immunol.*, **12**, 521-553.

Reimann, J. & Rudolphi, A. (1995) Co-expression of CD8 α in CD4$^+$ T cell receptor $\alpha\beta^+$ T cells migrating into the murine small intestine epithelial layer. *Eur. J. Immunol.*, **25**, 1580-1588.

Rocha, B., Vassalli, P. & Guy-Grand, D. (1991) The Vβ repertoire of mouse gut homodimeric α CD8$^+$ intraepithelial T cell receptor α/β^+ lymphocytes reveals a major extrathymic pathway of T cell differentiation. *J. Exp. Med.*, **173**, 483-486.

Rocha, B., von Boehmer, H. & Guy-Grand, D. (1992) Selection of intraepithelial lymphocytes with CD8 α/α co- receptors by self-antigen in the murine gut. *Proc. Natl. Acad. Sci. USA*, **89**, 5336-5340.

Shimizu, T., Kawamura, T., Miyaji, C., Oya, H., Bannai, M., Yamamoto, S., Weerasinghe, A., Halder, R.C., Watanabe, H., Hatakeyama, K. & Abo, T. (2000) Resistance of extrathymic T cells to stress and the role of endogenous glucocorticoids in stress associated immunosuppression. *Scand. J. Immunol.*, **51**, 285-292.

Sollid, L.M., Kvale, D., Brandtzaeg, P., Markussen, G. &

Thorsby, E. (1987) Interferon-γ enhances expression of secretory component, the epithelial receptor for polymeric immunoglobulins. *J. Immunol.*, **138**, 4303-4306.

Sonnenfeld, G., Cunnick, J.E., Armfield, A.V., Wood, P.G. & Rabin, B.S. (1992) Stress-induced alterations in interferon production and class II histocompatibility antigen expression. *Brain Behav. Immun.*, **6**, 170-178.

Taylor, C.T., Murphy, A., Kelleher, D. & Baird, A.W. (1997) Changes in barrier function of a model intestinal epithelium by intraepithelial lymphocytes require new protein synthesis by epithelial cells. *Gut*, **40**, 634-640.

Valente, G., Ozmen, L., Novelli, F., Geuna, M., Palestro, G., Forni, G. & Garotta, G. (1992) Distribution of interferon-γ receptor in human tissues. *Eur. J. Immunol.*, **22**, 2403-2412.

Van Damme, N., De Keyser, F., Demetter, P., Baeten, D., Mielants, H., Verbruggen, G., Cuvelier, C., Veys, E.M. & De Vos, M. (2001) The proportion of Th1 cells, which prevail in gut mucosa, is decreased in inflammatory bowel syndrome. *Clin. Exp. Immunol.*, **125**, 383-390.

Van Houten, N., Blake, S.F., Li, E.J., Hallam, T.A., Chilton, D.G., Gourley, W.K., Boise, L.H., Thompson, C.B. & Thompson, E.B. (1997) Elevated expression of Bcl-2 and Bcl-x by intestinal intraepithelial lymphocytes: resistance to apoptosis by glucocorticoids and irradiation. *Int. Immunol.*, **9**, 945-953.

Wyllie, A.H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature*, **284**, 555-556.

Yamada, K., Kimura, Y., Nishimura, H., Namii, Y., Murase, M. & Yoshikai, Y. (1999) Characterization of $CD4^+CD8\alpha\alpha^+$ and $CD4^-CD8\alpha\alpha^+$ intestinal intraepithelial lymphocytes in rats. *Int. Immunol.*, **11**, 21-28.

Yamamoto, S., Russ, F., Teixeira, H.C., Conradt, P. & Kaufmann, S.H. (1993) Listeria monocytogenes-induced gamma interferon secretion by intestinal intraepithelial γ/δ T lymphocytes. *Infect. Immun.*, **61**, 2154-2161.

Yoshikai, Y. (1999) The interaction of intestinal epithelial cells and intraepithelial lymphocytes in host defense. *Immunol. Res.*, **20**, 219-235.

Youngman, K.R., Fiocchi, C. & Kaetzel, C.S. (1994) Inhibition of IFN-γ activity in supernatants from stimulated human intestinal mononuclear cells prevents up-regulation of the polymeric Ig receptor in an intestinal epithelial cell line. *J. Immunol.*, **153**, 675-681.

Zhang, D., Kishihara, K., Wang, B., Mizobe, K., Kubo, C. & Nomoto, K. (1998) Restraint stress-induced immunosuppression by inhibiting leukocyte migration and Th1 cytokine expression during the intraperitoneal infection of Listeria monocytogenes. *J. Neuroimmunol.*, **92**, 139-151.