CHANGES IN LYMPHOCYTE SUBPOPULATIONS IN OTSUKA LONG-EVANS TOKUSHIMA FATTY RATS

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ABSTRACT

The changes in the thymus, spleen and adrenal glands associated with age from 5 weeks to 12 weeks old and lymphocyte subpopulations in the thymus and spleen were examined in the new strain of diabetic rats, Otsuka Long-Evans Tokushima Fatty (OLETF). Effects of restraint stress were also compared with those in the control strain, Long-Evans Tokushima (LETO) rats. The body weight, and weights of spleen and adrenal glands significantly increased with age in both strains, while the weight of the thymus in OLETF rats decreased although it increased in LETO rats. The percentage of CD4⁺CD8⁻ increased and CD4⁺CD8⁺ decreased in the thymus with age in both strains, and these changes were significantly enhanced in OLETF rats. The numbers of cells positive for nick end-labeling in the thymus were greater in OLETF than in LETO rats as shown by histological examination, although no significant difference was observed in the spleen or adrenal gland. The changes in lymphocyte subpopulations in the spleen, and the plasma corticosterone levels associated with age were comparable for both strains. The responses of these factors to restraint stress were not significantly different between the two strains. It has been suggested that the changes in T cell subpopulations in the thymus of OLETF rats may be simply induced by the enhanced apoptosis associated with age.

Otsuka Long-Evans Tokushima Fatty (OLETF) rats were developed as a model of non-insulin-dependent diabetes mellitus (NIDDM) (15). The characteristic features of OLETF rats are late onset of hyperglycemia at about 18 weeks old, followed by insulin deficiency at about 65 weeks old (15). Histological examination revealed (15) mild to moderate lymphocyte infiltration in or around almost all pancreas islets in OLETF rats at 6–20 weeks old. The islet structure was normal and insulin granules were identified by immunohistochemical staining

and the islets became hyperplastic at 20–40 weeks, then atrophied at >40 weeks old. However, the actual mechanism of NIDDM in OLETF rats is still unknown although multiple genetic abnormalities have been proposed to be responsible for the phenotype (9, 10, 15).

We recently found (5, 7) that OLETF rats lack cholecystokinin (CCK)-A receptor because of a genetic abnormality although the expression of CCK-B receptor was normal. OLETF rats revealed several abnormal functions; hyperphagia (20), decrease in exploratory behavior (16), impaired response of insulin and pancreatic enzyme secretion (6, 21) and retardation of pancreatic growth (22, 23). These might influence or promote the manifestation of NIDDM, but the disrupted CCK-A receptor itself could not be solely responsible for

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NIDDM in this model.

It has been shown (17, 18, 26) that stress influences hormonal balance and the immune system, and is one of the factors involved in NIDDM. Normal immune function can decline with age in mammalian species, and this decline occurs mainly in T cell-dependent immune functions (19). The thymus starts to involute at around puberty before the onset of decline of T cell-dependent immune functions (11–13, 18). In the present study, we examined whether OLETF rats show immune disturbance and whether the immune response to restraint stress in OLETF rats was different from that in normal controls. The lymphocyte subpopulations in the thymus and spleen, the histology of these organs and adrenal glands, and the plasma corticosterone levels were examined at 5 and 12 weeks old, during which period NIDDM is not manifested, although the infiltration of lymphocytes in the islets has been observed (15). Moreover, the responses to restraint stress of these factors were also compared with control Long-Evans Tokushima (LETO) rats at 12 weeks of age.

MATERIALS AND METHODS

Animals

Male OLETF and LETO (control) rats were obtained at 4 weeks of age from Tokushima Research Institute, Tokushima, Japan and were fed commercial rat chow (CRF-1). They were maintained in a vivarium at the Tokyo Metropolitan Institute of Gerontology, in a controlled environment at $23 \pm 2^\circ\text{C}$ under a 12-h light/12-h dark photocycle.

To examine the changes associated with age, male OLETF and LETO rats at 5 and 12 weeks old were sacrificed with a guillotine at 9 00–11 00 AM without fasting or any treatment, respectively ($n = 6$ for each strain). The thymus, spleen and adrenal glands were removed and weighed. Three animals were used for flow cytometric analysis to examine lymphocyte subpopulations in the thymus and spleen, and the rest were used for histological examinations. The blood was centrifuged at 3,000 rpm for 15 min at 4°C , the plasma was separated and frozen at -80°C until corticosterone assay. The plasma corticosterone concentrations were measured using RIA kit (Diagnostic Products, Los Angeles, CA).

To examine the effects of stress, 5 OLETF and 4 LETO rats were used at 12 weeks old, and 3 ani-

mals of each strain were used for flow cytometric analysis. The animals were restrained in a small cage made of wire gauze for 17 h. Then, the animals were released, and sacrificed after an additional 24 h with free access to food and water, because the suppressive effect of restraint stress on the immune system in mice appeared to be most prominent at this time (17, 18).

Flow Cytometric Analysis

Flow cytometric analysis was performed as described previously (17, 18, 27, 28). Briefly, cell suspensions were directly stained with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled monoclonal antibodies (mAb) and washed with phosphate-buffered saline containing 2% FBS (PBS-FBS). The stained cell suspensions were analyzed using a flow cytometer (EPICS-Elite; Coulter, Hialeah, FL). Monoclonal antibodies to rat lymphocytes were directed against CD3, CD45R, CD4, CD8, and were purchased from PharMingen (San Diego, CA).

Morphological Examination

The tissues were immersed in 10% formaldehyde solution for hematoxylin-eosin (HE) staining.

For the thymus, paraffin blocks were used for 3'-OH nick end-labeling studies (TUNEL method) with an Oncor Apop TagTM In Situ Detection Kit (Oncor, Gaithersburg) (8, 14, 24). Briefly, sections (3–4 μm) were mounted on polylysine-coated slides, deparaffinized in xylene, and dehydrated in absolute ethanol for 10 min. After rinsing in 50 mM phosphate buffer, pH 7.4, containing 20 mM NaCl (PBS), the slides were treated with proteinase K (20 $\mu\text{g}/\text{ml}$ in PBS) for 10 min at room temperature and incubated with 2% hydrogen peroxide diluted in PBS for 5 min to neutralize endogenous peroxidase activity. After rinsing with equilibration buffer, the sections were treated with terminal deoxynucleotidyl transferase and digoxigenin-uridine triphosphate for 60 min at 37°C then with antidigoxigenin peroxidase for 30 min at 37°C in a moisture chamber. The sections were then incubated with peroxidase-conjugated streptavidin (Nichirei, Tokyo) for 3 min at room temperature. After several rinses in PBS, the slides were stained to visualize the nick end-labeling with diaminobenzidine as the chromogen, rinsed in water, and counterstained with methyl-green or hematoxylin.

Table 1 *Wet Weights of Thymus, Spleen and Adrenal Glands in OLETF and LETO Rats at 5 and 12 Weeks Old (mg)*

	OLETF	LETO
<5 weeks old>		
Thymus	388.0 ± 10.9 (3.0 ± 0.1)	401.6 ± 16.2 (3.3 ± 0.2)
Spleen	594.6 ± 52.9 (4.6 ± 0.3)	493.6 ± 31.6 (4.0 ± 0.1)
Adrenal glands	27.2 ± 1.1 (0.19 ± 0.01)	25.1 ± 0.1 (0.24 ± 0.02)
	(n = 6)	(n = 6)
<12 weeks old: control>		
Thymus	357.4 ± 35.4 (0.9 ± 0.1*)	460.1 ± 18.6 (1.5 ± 0.1)
Spleen	758.5 ± 25.8 (1.9 ± 0.1)	686.7 ± 18.0 (2.2 ± 0.03)
Adrenal glands	40.7 ± 1.6 (0.11 ± 0.01)	39.8 ± 1.5 (0.13 ± 0.004)
	(n = 6)	(n = 6)
<12 weeks old: restraint stress>		
Thymus	266.5 ± 10.7 (0.8 ± 0.1)	343.8 ± 24.4 (1.2 ± 0.1)
Spleen	692.4 ± 20.9 (2.0 ± 0.1)	618.4 ± 11.7 (2.0 ± 0.02)
Adrenal glands	44.6 ± 0.2 (0.12 ± 0.004)	49.9 ± 0.1 (0.16 ± 0.003)
	(n = 5)	(n = 4)

The wet weights of adrenal glands are shown as the sum of right and left glands. The values in parentheses are ratios per body weight (mg/g).

*Indicates a significant difference from the corresponding value of LETO rats.

Statistical Analysis

Values are expressed as means ± SE. The results were analyzed by two-way analysis of variance, followed by Fisher's PLSD, respectively. A *P* value of 0.05 or less was considered significant.

RESULTS

Changes in Wet Weights and Results of Histological Examination of Thymus, Spleen and Adrenal Glands with Age

The body weights were 129.2 ± 6.5 g for OLETF and 124.7 ± 8.1 g for LETO at 5 weeks old, 395.5 ± 8.1 g for OLETF and 317.0 ± 5.5 g for LETO at 12 weeks old (mean ± SE). OLETF were significantly heavier than LETO rats at 12 weeks old.

The wet weights of adrenal glands were not significantly different regardless of age or strain (Table 1). The spleen of OLETF rats was significantly heavier than that of LETO rats, although the ratio per body weight was comparable for both strains. The wet weight of the thymus at 12 weeks old was significantly lower in OLETF than LETO

rats, and the ratio to body weight was also significantly lower in OLETF rats at 12 weeks old, whereas these were comparable for both strains at 5 weeks old.

On histological examination of the thymus, nick-end labeling positive cells were defined as those with brown staining of the nucleus. Color photographs (×100 magnification) were taken from histologically representative areas of thymic tissue in the two groups (Fig. 1). A minimum of 1,500 cortical cells in randomly selected areas was counted on the photographs for calculation of apoptosis labeling index. The apoptotic labeling index was calculated as the percentage of cells undergoing apoptosis. To avoid false positivity of necrotic cells, serial sections stained with HE were also observed. The numbers of cells positive for nick end-labeling were significantly higher in OLETF than LETO rats; 24.4 ± 2.5, mean ± SE for OLETF vs, 14.3 ± 1.6 for LETO rats, 8 examinations for each group, *t* = 3.4, *P* < 0.005.

There were no pathological findings by HE staining in other organs at any age examined.

Fig. 1 Histological examination of the thymus, nick-end labeling positive cells were defined as those with brown staining of the nucleus. $\times 100$

Changes in Lymphocyte Subpopulations in the Thymus and Spleen Associated with Age

The percentages of $CD4^+CD8^-$, $CD4^-CD8^+$ and $CD4^-CD8^-$ thymocytes in the thymus significantly increased ($F = 55.4$, 12.5 and 19.4 , $P < 0.01$) and those of $CD4^+CD8^+$ significantly decreased with age in both strains ($F = 68.2$, $P < 0.01$) (Fig. 2). The increase in $CD4^+CD8^-$ and decrease in $CD4^+CD8^+$ thymocytes of OLETF rats with respect to age were significantly different from

those of LETO rats; the interaction F of age and strain were 7.33 ; $P < 0.03$ and 8.4 ; $P < 0.02$, respectively (Fig. 2). The changes in percentages of $CD4^+CD8^-$ and $CD4^-CD8^-$ thymocytes associated with age were not significantly different between the two strains; the interaction F was 3.8 and 0.07 , $P < 0.8$.

In the spleen cells, the percentages of B cells ($CD45R^+$ cells) significantly decreased and those of T cells ($CD3^+$ cells) significantly increased associated with age, while there was no significant

Fig. 2 Percentage of thymic lymphocyte at 5 and 12 weeks old. All values were significantly changed with respect to age, and the values marked with asterisks were significantly different from the corresponding value of LETO rats by Fisher's PLSD.

difference between the two strains; the interaction F with respect to age and strain were 0.2; $P > 0.6$ and 3.4; $P > 0.1$, respectively (Fig. 3). The percentages of T cell subpopulations also increased significantly with respect to age and strain, whereas the CD4/CD8 ratio was not significantly different with respect to strain or age (the interaction $F = 0.65$, $P > 0.4$).

The Plasma Corticosterone Concentrations

The plasma corticosterone concentrations were 111.7 ± 28.9 pg/ml, (mean $\pm$ SE) for OLETF and 140.1 ± 73.4 pg/ml for LETO at 5 weeks old, 112.6 ± 58.2 pg/ml for OLETF and 198.7 ± 24.3 pg/ml for LETO rats for 12 weeks old. There were no significant differences with respect to age or strain.

Effect of Restraint Stress

The body weights significantly decreased after

restraint stress, and were 363.6 ± 10.8 g for OLETF and 313.5 ± 5.9 g for LETO rats at 12 weeks of age. The wet weights of spleen and thymus decreased significantly, while those of adrenal glands were not changed after restraint stress, but there was no significant difference between the two strains (Table 1). The interaction F values with respect to strain and treatment were 3.1, $P > 0.09$ for body weight, 1.2, $P > 0.29$ for spleen, 0.2, $P > 0.6$ for thymus and 3.9, $P > 0.06$ for adrenal glands.

The mean total cell numbers in the spleen were 59.7×10^7 for OLETF-control, 54.1×10^7 for OLETF-restraint, 62.1×10^7 for LETO-control and 68.7×10^7 for LETO-restraint, and those in the thymus were 91.1×10^7 for OLETF-control, 71.6×10^7 for OLETF-restraint, 147.1×10^7 for LETO-control and 103.9×10^7 for LETO-restraint. The percentages of CD4⁺CD8⁻ and CD4⁻CD8⁺ thymocytes significantly increased following restraint stress ($F = 9.2$ and 9.9 , $P < 0.02$, respectively) (Fig. 4). However, the interaction effects of strain

Fig. 3 Lymphocyte subpopulations in the spleen of rats at 5 and 12 weeks old

and treatment were not statistically significant ($F = 0.007$ and 2.88 , $P > 0.1$, respectively). The percentage of $CD4^+CD8^+$ thymocytes was significantly decreased, while that of $CD4^-CD8^-$ was not changed by stress ($F = 9.2$, $P < 0.02$ and $F = 0.02$, $P > 0.9$, respectively), but the interaction F values were not significant (0.06 and 0.75 , $P > 0.5$).

The percentages of B cells, T cells and T cell subpopulations ($CD4$ and $CD8$ T cells) in the spleen was not influenced significantly by restraint stress (the interaction with respect to strain and treatment $F = 0.23$, $P > 0.6$, $F = 2.3$, $P > 0.1$, 0.34 , $P > 0.5$, 2.46 , $P > 0.1$, respectively) (Fig. 5).

DISCUSSION

The body weight and weights of spleen and adrenal glands increased associated with age in both strains. The body weight of OLETF rats at 12 weeks old was significantly heavier than that of LETO rats as reported previously (7, 16, 20, 21), while there were no differences in terms of weights of spleen, thymus or adrenal gland or organ weight/body weight at 5 weeks old between the two strains. The thymus weight in OLETF rats decreased with age and apoptosis was enhanced at 12 weeks of age, although that of LETO rats increased (Table 1). Thymus weight usually increases until 10 weeks old in Wistar and Fisher-344 rats, and continues increasing until 12 weeks old in Sprague-Dawley rats (data from Nippon SLC, Shizuoka).

The size of the thymus is known to be influenced by sexual hormones during puberty before the onset of decline of T cell-dependent immune functions (12). Moreover, it has been shown (3, 12, 26) that glucocorticoid, adrenocorticotrophic hormone (ACTH) and sex hormones decrease the thymic gland and $CD4^+CD8^+$ subpopulation. However, in the present study, the plasma corticosterone level was not significantly different between both strains, although we did not measure the responses of corticosterone levels during restraint stress in this study. Although we have found several abnormalities due to the lack of CCK-A receptors in OLETF rats (3, 5, 7, 15, 20–23), there have been no studies of the relationship between CCK-A receptor and cellular immune function or function of the thymus. However, there was a report (2) that human T lymphoblastoid Jurkat cells possess CCK-B receptors. Therefore, it does not seem likely that the decreased size of the thymus in OLETF rats is due to plasma corticosterone level or to the lack of CCK-A receptors.

The changes in percentages of lymphocyte subpopulations associated with age were in the same direction as reported previously (11–13, 19) in both strains regardless of the size of the thymus or spleen (Figs. 1 and 2). The decrease in percentage of $CD4^+CD8^+$ cells (intermediate stage cells in the thymus) and increase in $CD4^-CD8^-$ cells (more mature cells) in the thymus associated with age were enhanced significantly in OLETF rats (Fig. 1), although changes in $CD4^-CD8^-$ (the most immature cells) and $CD4^-CD8^+$ (the more mature cells) were comparable for both strains. These enhanced changes in $CD4^+CD8^+$ and $CD4^-CD8^-$

Fig. 4 Percentages of thymic lymphocytes before and after restraint stress to rats at 12 weeks old

cells in OLETF rats were compatible with the enhanced apoptosis and decrease in size of the thymus (8, 11–13, 18). On the other hand, the lymphocyte subpopulations in the spleen were not significantly different between the two strains or ages examined (Fig. 2) in addition to there being no significant differences in the changes in spleen weight. Therefore, the changes in T cell subpopulations in the thymus of OLETF rats may be the result of the faster decrease in thymus weight in these animals associated with age, simply induced by overnutrition and early growth because of hyperphagia (20).

Restraint stress induced decreases in body weight, thymus and spleen wet weight but no changes in weight of adrenal glands in both strains. It has been reported that physical stress changes the cellular composition of the thymus by increasing the percentages of CD4⁻CD8⁻, CD4⁻CD8⁺ and CD4⁺CD8⁻ thymocytes and by decreasing that of CD4⁺CD8⁺ thymocytes (1, 4, 25–28). Our results showed similar effects of restraint stress on the percentages of T cell subpopulations (Fig. 3). However, those in the spleen were not significantly influenced by restraint stress (Fig. 4). In previous studies in mice (27, 28), the percentage of CD4⁺CD8⁺ cells in the thymus and spleen were

significantly decreased one day after 17 h restraint stress. However, those in the spleen did not significantly decrease in either OLETF or LETO rats after restraint stress, and the responses to stress were not significantly different between the two strains. We examined these parameters at only one time point after restraint stress, and it may be necessary in future studies to analyze the changes at multiple time points because the responses in rats might be different from those in mice.

We did not examine the lymphocyte subpopulations after the onset of NIDDM in this study, but it will be necessary to examine the changes in immune functions before and after the manifestation of NIDDM in the future.

In conclusion, the thymus in OLETF rats decreased in size and showed enhanced apoptosis at 12 weeks of age, and the changes in T cell subpopulations in the thymus of OLETF rats associated with age were different from those in normal animals. However, these changes seemed simply to be due to early growth and may have little role in the manifestation of NIDDM in these animals.

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Fig. 5 Lymphocyte subpopulations in the spleen before and after restraint stress to rats at 12 weeks old

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