Effect of Neonatal Gonadectomy on Lymphocytes Population and Blast Formation in Aged Rats

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ABSTRACT. In male rats gonadectomized at birth, the blast formation reaction of lymphocytes was significantly lower at 114 weeks of age, than that of non-gonadectomized controls, whereas the lymphocyte subpopulation was similar. The thymus of neonatally castrated rats of age 114 weeks was histologically normal, decreasing in weight to 68% of that of non-castrated rats.—KEY WORDS: aged rat, gonadectomy, lymphocyte.

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Lymphocytes in aged man or mouse have lower reactivity to mitogens, such as phytohemagglutinin (PHA) and concanavalin A (Con A) [7, 9], supposedly due to degraded IL-2 receptor and depressed IL-2 productivity with ageing [2–6, 15]. The lowered cellular immunity of aged individuals might be associated with involution of the thymus [8, 12, 19]. At this laboratory we found that a male pseudohermaphrodite rat congenitally lacked androgen secretion showing persistence of the thymus after growing [10]. The neonatally gonadectomized and aged rats were reported to have the histologically normal thymus without regression with age [24]. In this study, neonatally castrated and aged male rats were examined for the lymphocyte subpopulation and its reaction to mitogen as compared with those of non-gonadectomized controls.

Male Wistar-Imamichi rats were bred at Imamichi Institute for Animal Reproduction. Gonadectomy was made under ether anesthesia on day 0 of age prior to sucking. Animals were weaned on day 21 of age and transferred to a breeding chamber (24°C, relative humidity of 55 ± 10%, and ventilation of 12- to 15 changes per hour). The room were lightened for 14 hrs (5:00 ~ 19:00) a day. Animals were freely given commercial pellets (MM-3; Funabashi Farm Ltd.) and tap water. Blood was collected from the jugular vein under ether anesthesia, and 0.1 mg per 1 ml EDTA-2K (Autosep; Terumo, Tokyo) was added as anticoagulation.

Four monoclonal mouse IgG antibodies, MAS 010, MAS 1131, MAS 041 and MAS 043 (Sera-Lab., U.S.A.) were used for identifying rat lymphocyte subsets, helper T cells [25], suppressor or cytotoxic T cells [17], peripheral B cells and monocytes [18], respectively.

In flow cytometry, 100 µl of the whole blood of test animals was mixed with an equal volume of 1:50 dilution of each monoclonal antibody in PBS containing no Ca²⁺ and Mg²⁺, pH 7.4–7.6, and the mixture was allowed to stand for 30 min at 4°C. After washing twice with PBS, 100 µl of 1:40 dilution of fluorescein isothiocyanate (FITC)-labeled anti-mouse caprine IgGs (Tago, U.S.A.) and 25 µl of rat plasma were added, and the mixture was kept for 30 min at 4°C. After washing with PBS, 5 µl of fetal calf serum (Filton, U.S.A.) and 2 ml of hemolytic reagent

(NH₄Cl 4 g/l, KHCO₃ 1 g/l, EDTA-4Na 0.037 g/l) were added to the solution, which was then allowed to stand at a room temperature for 3 to 5 min. The sample was subjected to flow cytometry using Orthospectrum III (Ortho Diagnostic Systems, U.S.A.) [13] with three light parameters, forward scattered, 90° scattered and green fluorescent at 515 to 620 nm. The image monitoring was set up for the percentage of positive cells to be less than 1% of the total cell number.

Lymphocytes were isolated by the gravity centrifugation using Ficoll-conray [1] and used an isolation reagent described previously [11]. To 100 µl of lymphocyte suspension (1 ~ 2 × 10⁷/ml), 850 µl of a culture medium and 50 µl of mitogen solution (PHA-M: 0.5 g/ml, Con A and PWM: 2 mg/ml) were added and the mixture was incubated at 37°C for 96 hr in a 5% CO₂ atmosphere in air [11]. The culture medium was RPMI 1640 (Nissui, Tokyo) supplemented with 4 mg/ml of sodium bicarbonate (Wako Pure Chemicals, Tokyo), 25 mM of N-Hydroxyethylpiperazine-N'-2-ethansulfonic acid (HEPES; SIGMA, U.S.A.), 100 units/ml penicillin G potassium salt (Meiji, Tokyo), 0.2 mg/ml streptomycin sulfate (Meiji, Tokyo) and 0.1 ml/ml rat serum. The rat serum was from 10 week-old male Wistar-Imamichi SPF rats anesthetized with CO₂ and heated at 56°C for 30 min.

As mitogens, phytohemagglutinin-M (PHA-M; 50 mg/ml, Difco, U.S.A.), concanavalin A (Con A; SIGMA, U.S.A.) and pokeweed mitogen (PWM; Bio-sys S.A., U.S.A.) were used at 0.05 ml/ml and to 10 µg/ml in culture medium, respectively.

To see glucose concentration, lymphocyte culture was centrifuged at 3,000 rpm for 10 min, and the supernatant was examined for glucose concentration using an RS 1100 analyzer (JEOL, Tokyo) [11]. The glucose consumption was then converted to a stimulation ratio (GCT-SR) for blast formation according to the following formula: GCT-SR = (Gcont-Gstim) / Gcont × 100%

GCT-SR: glucose consumption test-stimulation ratio,
Gcont: glucose concentration in the control culture,
Gstim: glucose concentration in the stimulated culture.

The wet thymuses were weighed at autopsy using an electronic balance (AE-163, Mettler, Switzerland). The organs were fixed in 10% neutral buffered formalin, and sections were made and stained with hematoxylin-eosin for microscopy.

Data from the laser flow cytometry as well as GCT-SR

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Table 1. Lymphocyte subset ratio

Subset ^{a)}	114 weeks of age		10 weeks of age
	neonatally castrated	non-castrated	non-castrated
T	66.8±9.62 ^{b)}	61.0±7.16	65.2±6.24
Th	43.7±3.88	42.8±7.17	47.5±5.75
Ts/k	15.3±6.41	12.7±3.61	9.7±2.42
Th/(Ts/k)	3.5±2.16	3.6±1.06	5.0±0.88
B and M	9.0±4.73	13.7±6.92	11.0±2.53

a) T: peripheral T. Th: helper T, Ts/k: suppressor or killer T, B and M: B cell and monocyte.

b) % of total lymphocytes: mean ± S.D. (6 of animals).

Table 2. Blast formation of lymphocytes based on glucose consumption test

Mitogen	114 weeks of age		10 weeks of age
	neonatally castrated	non-castrated	non-castrated
PHA-M	24.1±6.98 ^{a)***}	5.3±3.20 ^{**}	43.4±8.75
Con A	13.5±3.78 ^{**}	8.8±6.71 ^{***}	49.5±15.54
PWM	9.8±9.32	7.6±5.65	5.8±4.53

a) % of GCT-SR values: Mean ± S.D. (6 of animals)

***: Significantly differed from values for young (intact) rats where $p < 0.001$.

values and body and thymus weights were analyzed of variance based on the alternate two-way classification. Factors and interaction between two statistically significant factors, leveled average values were compared according to Tukey's method [22].

At 114 weeks of age, neonatally castrated aged rats and non-castrated controls showed survival rates of 83.3% and 55.5%, respectively.

As shown in Table 1, the total T cells of the castrated rats and non-castrated controls were 66.8 ± 9.62 and $61.0 \pm 7.16\%$ of lymphocytes, respectively, at 114 weeks of age. The helper T lymphocytes were 43.7 ± 3.88 and $42.8 \pm 7.17\%$ in castrated and non-castrated rats, respectively, whereas the suppressor/cytotoxic T cells were 15.3 ± 6.41 and $12.7 \pm 3.61\%$, respectively. The ratio of the helper and suppressor/cytotoxic T cells were 3.5 ± 2.16 and 3.6 ± 1.06 , respectively. The B cells and monocytes of neonatally castrated and non-castrated controls were 9.0 ± 4.73 and $13.7 \pm 6.92\%$, respectively, at 114 weeks of age.

As presented in Table 2, the GCT-SR values from the lymphocyte culture with 0.05 ml/ml PHA-M were $24.1 \pm 6.98\%$ and $5.3 \pm 3.2\%$ in neonatally castrated rats and non-castrated controls, respectively, at 114 weeks of age, and the neonatally castrated rats showed a significantly higher value as compared with non-castrated controls ($p \leq 0.001$). However the both values were significantly lower than that of intact young rats ($43.4 \pm 8.75\%$). Similar results were obtained with the GCT-SR values from the lymphocyte cultures with 10 $\mu\text{g/ml}$ Con A. In the culture with 10 $\mu\text{g/ml}$ PWM, difference was not significant among the three groups of rats. The thymus weight of

neonatally castrated rats was 68% of that of 10-week-old intact rats.

The decline of immune functions with decreased lymphocyte functions has been reported in aged man or rats [20]. The lymphocytes is known to grow in the thymus and to be distributed to the periphery when matured. The senile decreased immune functions of T cells is supposed to be in parallel with age-associated degeneration of thymus [9]. On the other hand, gonadectomized and aged animals were reported to have the relatively preserved thymus [10, 24]. Sex hormones such as estradiol or testosterone is known to cause delayed thymus regression [16, 21].

In the present study, the circulating lymphocyte subsets were not significantly different between the neonatally castrated rats and controls. However, the neonatally castrated rats showed significantly higher values of blast formation of lymphocytes in cultures with 2.5 mg/ml PHA-M as compared with controls. The neonatally castrated rats showed a significantly lower glucose consumption, indicating that the reactivity of their lymphocytes to mitogen was retained. Thus the sex hormones might be a key factor for regulating immune functions.

No significant difference was seen in blast formation in culture with PWM between neonatally castrated rats and non-castrated controls. Of the effect of neonatal castration on B cell functions different results have been obtained [7, 9], and further investigation will be required.

The declined ability of T cell blast formation in aged rats reported by Nikki *et al.* [20] was more remarkable than in the present study, possibly because of using different strains of rat. The aging process has been reported to be significantly variable among mouse strains [14].

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