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COMBINED GRAFTING OF BONE MARROW AND THYMUS, AND SEQUENTIAL MULTIPLE THYMUS GRAFTINGS IN VARIOUS STRAINS OF MICE. THE EFFECT ON IMMUNE FUNCTIONS AND LIFE SPAN

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SUMMARY

The combined grafting of young bone marrow and newborn thymus performed in old mice was effective in restoring the impaired immune functions, but the same treatment performed in middle-aged adult mice had no effect on the life span of C3H/MTV female mice. Sequential multiple newborn thymus graftings starting at young adult age were effective in enhancing immunological functions, delaying the onset of tumor and extending the survival rate to certain degree in the first half of the experimental course in both C3H/MTV as well as C57BL/6 mice, but these effects were not observed in the latter half of the experimental course. It was suggested that multiple newborn thymuses sequentially implanted into the peritoneal cavity underwent atrophy and these atrophic thymuses had a suppressive effect on the host immune system. In autoimmune prone B/WF1 mice, however, the combined grafting of young bone marrow and newborn thymus resulted in suppression of antibody formation to SRBC, and single grafting of either young bone marrow or newborn thymus resulted in a trend of increase in the antibody formation to SRBC. The sequential multiple newborn thymus graftings in B/WF1 mice brought about aggravation of kidney diseases and shortening of the mean life span. On the contrary, administration of thymosin in B/WF1 mice resulted in amelioration of kidney disease and elongation of the mean life span.

Key words: Thymus grafting; Bone marrow grafting; Thymosin; Immune functions; Life span; Mouse

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Abbreviations: PHA, phytohemagglutinin; Con A, concanavalin A; LPS, lipopolysaccharide; MNTG, sequential multiple newborn thymus grafting; MNSG, sequential multiple newborn spleen grafting; SRBC, sheep red blood cells; B/WF1 [NZB/NZW]F_1; BMT, bone marrow transplantation; MLS, mean life span; PFC, plaque forming cells.

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INTRODUCTION

The immune activity is known to decline with age in man and most mammals [1,2] although the onset, magnitude and rate of the decline are different by species, strain and immunological indices. The decline mainly occurs in T cell dependent immune functions, and this can be mainly ascribed to thymic involution which starts at around puberty [3]. The rapid decline of T cell recruitment after the birth is in striking contrast to the continuous recruitment of B cells and macrophages from the bone marrow throughout the life, and could be one major reason why T cell dependent immune functions is vulnerable to aging. In practice, the age-related decline of immune functions could be retarded in C57BL/6 mice by the sequential multiple graftings of newborn thymus performed every 2 months [4]. The declined immune activity in aged mice can be restored to the young level by the combined grafting of young bone marrow and newborn thymus [5]. In the present paper, three strains of mice underwent the following two treatments: (1) the sequential multiple graftings of newborn thymuses and (2) the combined grafting of young bone marrow and newborn thymus, and the effect of these treatments was examined in terms of the restoration of immune functions and the elongation of life expectancy.

MATERIALS AND METHODS

Mice

C57BL/6NCrJ were purchased from Charles River Japan (Kanagawa), and C3H/HeNJel-MTV (C3H/MTV) and [NZB/NZW]F_1 (B/WF1) were purchased from Japan Clea (Tokyo). All were female mice and reared in specific pathogen free (SPF) colony of Tokyo Metropolitan Institute of Gerontology. Pregnant mice were also purchased from companies mentioned above and used for donors of newborn thymus. In case of B/WF1 mice, thymus donors were DBA/2 mice which have the same H-2 with the recipients. C57BL/6NCrJ female mice (mean life span, 549 ± 27 days) develop high incidence of malignant lymphoma, starting at around 40 weeks of age and cumulative incidence is approximately 50% until 100 weeks of age [6]. C3H/HeNj-Mtv female mice (mean life span, 407 ± 10 days) develops mammary adenocarcinoma, starting at around 29 weeks of age and the cumulative incidence is 100% until 60 weeks of age. B/WF1 female mice (mean life span, 318 ± 19 days) develop autoimmune disease similar to systemic lupus erythematosus in human and die of mainly kidney disease.

Thymus grafting

In experiments of sequential multiple newborn thymus graftings, two lobes of newborn thymus from donor mice of syngeneic strain or of identical H2 histocompatibility antigen were injected into the peritoneal cavity by Argyle Medicut Cannula (Japan Sherwood, Tokyo). Newborn thymus grafting was performed every two

months in C57BL/6 and C3H/HeN mice, and every month in B/WF1 mice. For control groups, newborn spleen was grafted intraperitoneally in the same manner (MNSG). In the experiment of single grafting of thymus, newborn or old thymus was grafted under kidney capsule.

Combined grafting of young bone marrow and newborn thymus

Mice were irradiated at dose of 600 R and given bone marrow cells (5×10^6) from young syngeneic donors. They were then immediately grafted with two lobes of syngeneic newborn thymus under the kidney capsule.

Thymosin administration

In B/WF1 mice, i.p. injection of synthetic thymosin α-1 (10 μg/0.1 ml PBS) was performed twice a week.

Thymectomy

Thymectomy was performed in C57BL/6 mice at 4 weeks of age as described previously [3].

Mitogenesis

PHA, Con A and LPS responses of splenic lymphocytes were performed as described previously [3].

Statistics

Wilcoxon rank sum test was employed to test the difference in the mean life span between experimental groups. Student's *t*-test was employed to test the difference in immunological data between experimental groups.

RESULTS

Effect of combined grafting of bone marrow and thymus on the life span of C3H/MTV female mice

In the previous reports, it was shown that the combined grafting of young bone marrow and newborn thymus was effective in the restoration of immune function of middle aged and old BC3F1 mice which do not develop any specific age-related diseases. Thus, in the present study, it was examined whether or not the same treatment was effective in the prevention or retardation of the occurrence of mammary tumor in C3H/MTV mice and accordingly in the elongation of their life span. Mice were separated into four groups: A, Control without treatment, B, Young bone marrow transplantation only; C, Newborn thymus grafting only; D, Combined grafting of young bone marrow and newborn thymus. As mammary tumor in nulliparous C3H mice usually starts to occur at around 29 weeks of age, the treatment

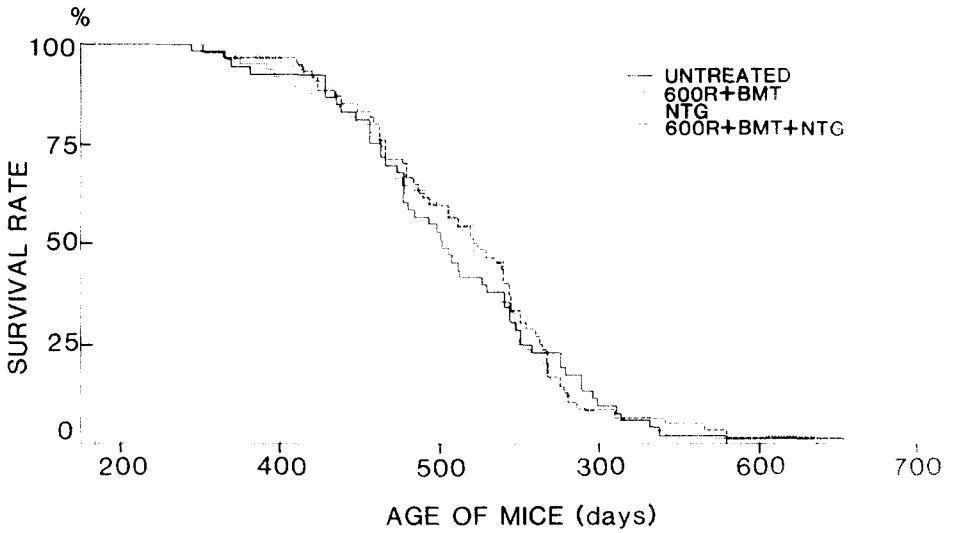

Fig. 1. Survival rate of C3H/MTV female mice treated with 4 different ways at the age of 25 weeks. *Untreated control* (continuous line; MLS, 407 ± 10 days). *600 R + BMT* 6.0 Gy total body iradiation followed by bone marrow transplantation (finely dotted line; MLS, 407 ± 10 days). *NTG* newborn thymus grafting (dotted line; MLS, 433 ± 12 days). *600 R + BMT + NTG*, 6.0 Gy total body irradiation followed by bone marrow transplantation and newborn thymus grafting (broken line; MLS, 417 ± 10 days).

was performed at 25 weeks (175 days) of age, approximately 4 weeks before the onset of the tumor, and the survival rate was observed thereafter.

As shown in Fig. 1, there was no significant difference in survival rate among four groups until about 450 days, and after 450 days a slight elongation of survival rate was observed in group C which had been treated with newborn thymus grafting alone ($P = 0.15$, between A and C).

TABLE I

SHORT-TERM EFFECT OF MULTIPLE NEWBORN THYMUS GRAFTING IN C3H/MTV MICE

	PFC/spleen *(× 10^3)*	*PHA* *(× 10^3)*	*Thy-1⁺ cells in spleen (× 10^7)*
Control	311	13.4	3.17
	(352—274)	(15.0—12.0)	(3.32—3.02)
Experiment	748	13.2	3.86
	(839—666)	(14.1—12.3)	(4.13—3.61)

Newborn thymus grafting was performed in C3H/MTV mice 3 times (3 m, 5 m, 7 m). Immunologic assay was performed at 2 months after the last treatment. Control mice were grafted with newborn spleen instead of newborn thymus. Numbers indicate geometric means of 15 samples with the range of 1 S.E. in parentheses.

The effect of sequential multiple newborn thymus grafting in C3H/MTV mice

In the first experiment, the sequential multiple newborn thymus grafting (MNTG) was performed in C3H/MTV female mice, every 2 months starting from 2 to 6 months old, 3 times in total, and some immunological functions were assessed at 7 months of age, 1 month after the last grafting. Although most of immunological functions in C3H/MTV at 7 months of age were still maintained at relatively high level, a significant increase was observed in the number of splenic T cells as well as in anti-SRBC antibody activity (Table I).

In the second experiment, MNTG treatment was performed every 2 months starting from 3 months of age throughout the life and life expectancy was observed. In this experiment, elongation of the life expectancy was observed after 350 days old (Fig. 2). It was revealed that the treatment could delay the onset of occurrence of the tumor resulting in the elongation of the mean life expectancy to a certain degree; i.e., the mean life span, 515 ± 20 days in experimental group and 465 ± 20 days in control group (P = 0.07). However, no difference was observed in the maximum life span between two groups.

The effect of sequential multiple newborn thymus grafting in aging C57BL/6 mice

Firstly, grafting of either newborn thymus or newborn spleen was sequentially performed in C57BL/6 mice every 2 months, 5 times in total, starting at 3 months

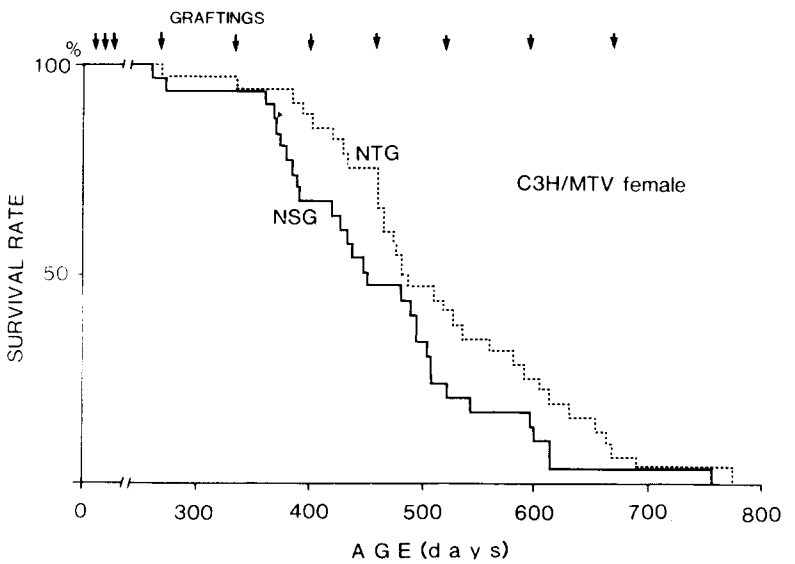

Fig. 2. Survival rate of C3H/MTV female mice, treated with sequential multiple graftings of newborn thymus (NTG, dotted line: MLS = 515 ± 20 days) or newborn spleen (NSG, solid line: MLS = 465 ± 19 days). P = 0.07. Graftings were performed every 2 months, starting at 3 months of age and ending at 21 months of age (10 times grafting in total).

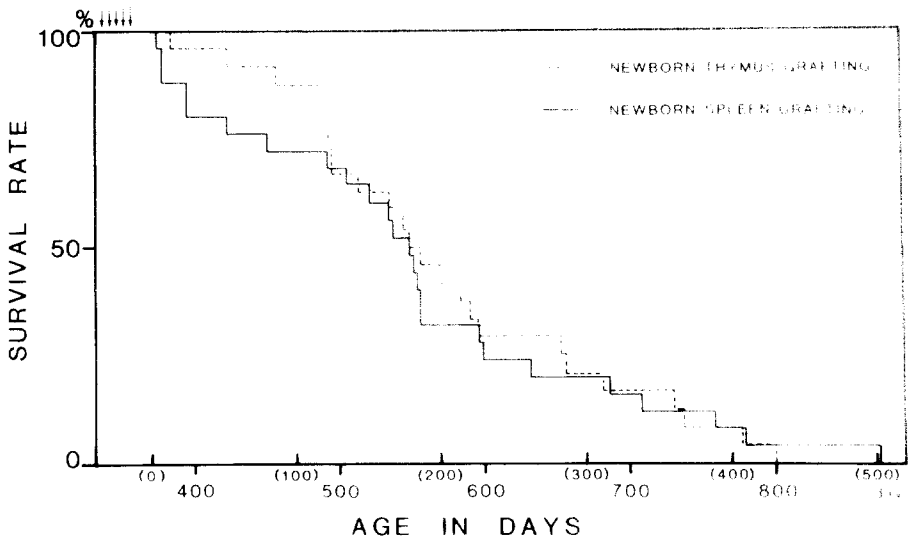

Fig. 3. Survival rate of C57BL/6 female mice, treated with multiple grafting of newborn thymus (dotted line: MLS = 548 ± 27 days) and newborn spleen (continuous line: MLS = 573 ± 23 days). Grafting was performed 5 times in total, starting from 3 months of age and ending 11 months. Improvement of survival was temporarily observed during approximately 100 days after the last grafting. But no difference in the final mean life span, 549 ± 27 days in control and 573 ± 23 days in experiment.

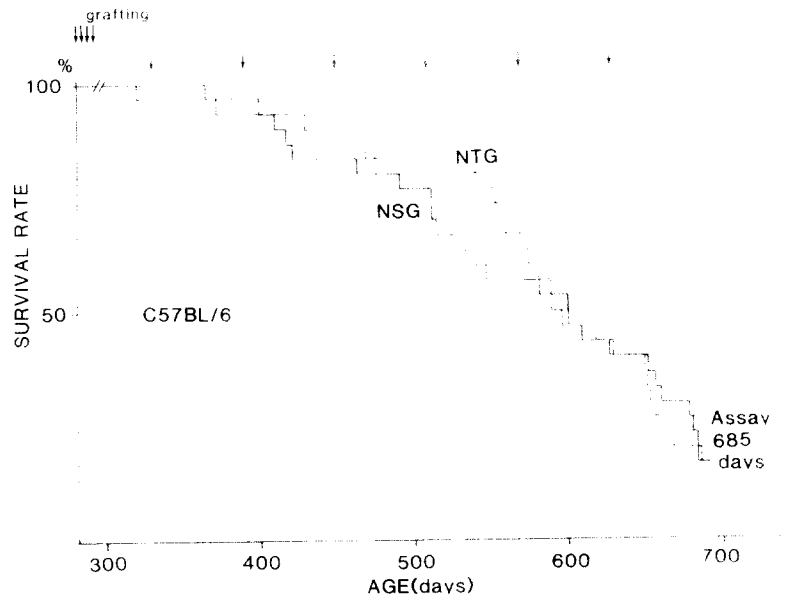

Fig. 4. Survival rate of C57BL/6 female mice, treated with sequential multiple graftings of newborn thymus (NTG, dotted line: 566 ± 19 days) or newborn spleen (NSG, solid line: 551 ± 21 days). Graftings were performed every 2 months, starting at 3 months of age and ending at 21 months (10 times graftings in total). At 685 days, all mice were sacrificed and their spleens were prepared for immunological assay.

and ending at 11 months of age, and the survival of both groups was observed. It was revealed that the elongation of life expectancy was temporarily observed during approximately 100 days after the last thymus grafting. However, there was no difference in the survival rate (the mean life span, 573 ± 23 days in experiment and 549 ± 27 days in control: $P = 0.5$) as well as the maximum life span between experimental and control group (Fig. 3).

In the second experiment, the grafting was performed every 2 months, 10 times in total, starting from 3 months and ending at 21 months of age (Fig. 4). A trend of elongation of life expectancy was observed between 300 and 600 days ($P = 0.14$), but absolutely no difference after the 9th grafting. Thus, at 685 days of age, about 1 month after the last grafting, all surviving mice were sacrificed and their immunological functions were examined. The experimental group with thymus graftings showed a slight increase in the percentage of splenic T cells with a significant increase of surface density of Thy-1 antigen, as compared with control. In terms of immune functions, however, no significant enhancement was observed in immune functions of aged mice of the experimental group (Table II).

Effect of the combined grafting of young bone marrow and newborn thymus on the immune response of B/WF1 female mice

B/WF1 female mice spontaneously develop a progressive SLE-like autoimmune disease in association with accelerated age-related decline of immune functions. In the first experiment, mice were separated into four groups and underwent four kinds of treatments. They were: A, Control without treatment; B, Young bone marrow transplantation only; C, Newborn thymus grafting only; D, Combined grafting of young bone marrow and newborn thymus. The treatment was performed at 12

TABLE II

LONG-TERM EFFECT OF MULTIPLE NEWBORN THYMUS GRAFTING IN C57BL/6 MICE

	PFC/spleen	*PHA*	*ConA*	*Thy-1⁺ cells %*	*Thy-1 density (mean channel)*
MNSG	1324 (1790—980)	679 (1026—449)	1289 (2109—787)	12.6 ± 3.7	128.2 ± 0.7*
MNTG	975 (1195—795)	1249 (1877—831)	2849 (3723—2181)	18.3 ± 5.4	136.4 ± 2.7*
Cont. (6 m)	870 582 (1 260 479 —601 290)	32 401 (34 285 —30 620)	56 416 (60 147 —52 916)	26.7 ± 2.9	135.0 ± 1.0

Sequential multiple newborn thymus graftings (MNTG) were performed every 2 months, from 3 to 21 months of age, 10 times in total. Survived mice at 685 days were sacrificed and used for immunological assessment. Sequential multiple newborn spleen graftings (MNSG) were performed in the similar manner as mentioned above and used as a control group. As young control, 6 months old mice were used.
*Indicates a significant difference ($P < 0.05$).

TABLE III

EFFECT OF GRAFTING OF NEWBORN THYMUS AND BONE MARROW IN NZB/WF1 FEMALE MICE

	(A) Control	*(B) 600R + BMT*	*(C) NTG*	*(D) 600R + BMT + NTG*
	[18]	*[13]*	*[15]*	*[16]*
Body weight (g)	40.6 ± 1.6	40.6 ± 0.6	38.1 ± 2.4	39.1 ± 1.4
Anti-SRBC response PFC/10^6	89 700 (120 635—66 693)	174 377 (290 679—104 608)	106 050 (168 565—66 720)	25 553 (41 297—15 811)
LPS-induced autoantibody PFC/10^6	74 (102—54)	50 (95—26)	119 (152—93)	62 (92—42)
PHA response	7315 (12 234—4374)	8587 (14 610—5047)	10 017 (13 646—7353)	6377 (9483—4289)
LPS response	6587 (7841—5530)	6481 (10 321—4070)	6348 (10 862—3710)	6931 (8661—5544)

Bone marrow transplantation was performed after 600 R total body irradiation (600R + BMT). Newborn thymus grafting was performed under kidney capsule (NTG). Combined grafting of BMT and newborn thymus was performed first with 600R + BMT and subsequently with NTG. All treatments were performed at 3 months of age. Immunological assays were performed 6 months after the treatment. Numbers in parentheses, range of 1 S.E. of the geometric mean. Numbers in brackets, numbers of mice used in the groups.

weeks of age, and various immunological indices were examined about 6 months later. In terms of anti-SRBC antibody response, a significant decrease was observed in group D with combined grafting of young bone marrow and newborn thymus, but no significant difference was observed in groups B and C as compared with control group A. In terms of LPS-induced autoantibody, group C with newborn thymus grafting showed a trend of increase, and other groups B and D showed a trend of decrease. There was no significant difference in PHA and LPS responses (Table III).

Effect of sequential multiple newborn thymus grafting and administration of thymosin on disease and life span of B/WF1 female mice

The effect of the sequential multiple thymus grafting (MNTG) was tested in B/WF1 female mice and compared with that of the sequential multiple newborn spleen grafting (MNSG) and of thymosin administration. It was shown that MNTG promoted the development of autoimmune disease in terms of amount of proteinuria and shortened the life span. To the contrary, the treatment with thymosin α-1 which was given i.p. two times per week was effective in delaying the development of proteinuria and in elongating the life expectancy (Fig. 5). The mean life expectancy was 318 ± 19, 280 ± 9 and 342 ± 22 days in control (MNSG), MNTG and thymosin-

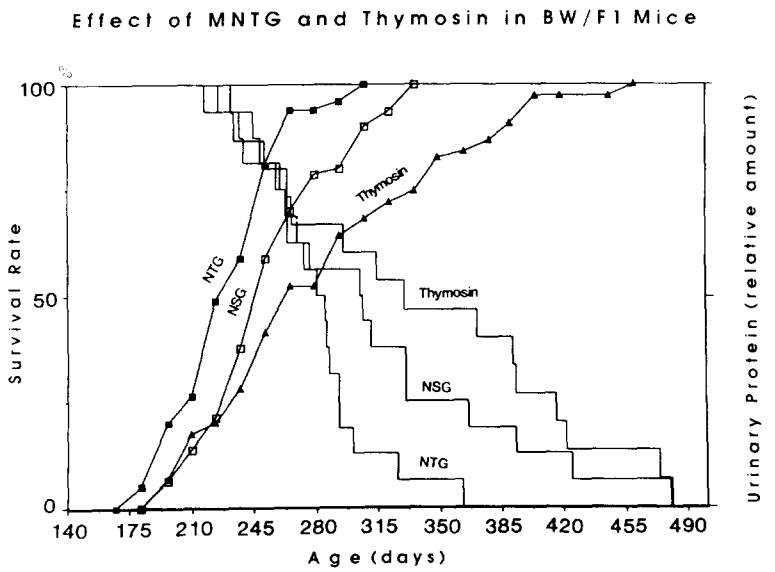

Fig. 5. Survival rate of B/WF1 female mice, treated with sequential multiple graftings of newborn thymus (NTG, closed squares: MLS = 280 ± 9 days), newborn spleen (NSG, open squares: MLS = 318 ± 19 days) or thymosin α-1 (closed triangles: MLS = 342 ± 22 days). Graftings were performed every month, starting at 2 months of age and ending at 11 months of age (10 times grafting in total). Thymosin α-1 (10 μg) was intraperitoneally given 2 times per week. The relative amount of urinary protein of each group was also shown as closed squares (NTG), open squares (NSG) and closed triangles (thymosin α-1).

treated groups, respectively. The difference between MNTG and thymosin-treated groups was nearly significant ($P = 0.055$).

Effect of newborn and old thymus grafts on the immune function of adult-thymectomized mice

The fact that sequential graftings of newborn thymuses more than 10 times were not effective in the restoration of immune functions of aging mice suggested that multiple atrophic thymuses implanted in the peritoneal cavity might negatively influence the immune functions of the recipients. To test this possibility, mice were thymectomized at 4 weeks of age and immediately treated in 3 ways: (i) control without treatment; (ii) grafting of one pair of newborn thymus and (iii) grafting of five pairs of old thymuses. Two months after the treatment, number and percentage of splenic T cells, and their mitogenic response were examined. It was shown that the number of splenic T cells and their subsets, and their mitogenic response of mice thymectomized at 4 weeks of age declined to about 50% as much as the level of age-matched controls. Only LPS response was comparable with that of control. Two months after the grafting of newborn thymus, a number of splenic T cells and their mitogenic response recovered to the control levels. By grafting of five pairs of old

TABLE IV

EFFECT OF YOUNG AND OLD THYMUS GRAFTS ON T CELLS IN ADULT THYMECTOMIZED MICE

	Control (Sham TX + Sham-TG)	*TX + Sham-TG*	*TX + NTG*	*TX + OTG × 5*
Splenic total cell count	1.71×10^8 (1.91—1.54)	1.17×10^8 (1.20—1.14)	1.69×10^8 (1.80—1.58)	1.87×10^8 (2.00—1.75)
T cells, number in spleen	5.76×10^7 (6.37—5.22)	2.81×10^7 (2.88—2.75)	5.93×10^7 (6.39—5.51)	5.18×10^7 (5.47—4.91)
PHA	30 853 (34 922—27 258)	19 228 (21 534—12 169)	30 722 (34 553—27 316)	7557 (9324—6124)
Con A	49 559 (52 155—47 092)	20 727 (23 265—18 466)	45 415 (52 177—39 529)	8480 (11 108—6474)
LPS	11 615 (13 310—10 316)	13 377 (14 664—12 203)	16 247 (17 916—14 733)	13 115 (14 526—11 841)
PFC/10^6 spleen cells	5438 (6448—4587)	1733 (1963—1530)	3169 (3843—2620)	1775 (1978—1592)

Male C57BL/6 mice were thymectomized at 4 weeks of age, and grafted with either one pair of newborn thymus (NTG) or five pairs of atrophic thymus at 24 months of age (OTG × 5). In one control group, mice were sham-thymectomized (sham-TX) and underwent sham-thymus grafting (sham-TG). In other control group, thymectomized mice underwent sham-thymus grafting. All assessments were performed 3 months after the treatment. Numbers indicate, geometric mean with 1 S.E. of the geometric mean in parentheses.

atrophic thymus, the number of splenic T cells recovered to the control level, but their mitogenic responses declined further than the level of thymectomized mice. The findings suggested that multiple old thymus grafts brought about suppressive effect on the proliferative capacity of whole splenic T cells, either by some suppressor cells or by factors (Table IV).

DISCUSSION

There are two aspects in the experiment of immunological restoration. One is how the impaired immune functions of old individuals can be restored to the level approaching to that of young ones. The second is what is the effect of the immunological restoration in aging individuals; e.g., is it possible to delay or prevent the onset of aging diseases, or to elongate the life span? Our previous study [8] indicated that the impaired immune functions could be partly ascribed to the imbalance of T cell subsets that work in immune functions as effector cells as well as regulatory cells. Thus, simple injection of young cells or grafting of young organs into old animals can not satisfactorily correct the imbalance of T cell subsets in them, although some beneficial effect can be temporarily obtained [9,10]. One method to correct the imbalance of T cell subsets in old individuals is the combined grafting of young bone

marrow cells and newborn thymus after removing old cells by whole body irradiation [5]. Another method is the prevention of progression of imbalance of T cell subsets by sequential grafting of newborn thymus starting from young age [4].

The combined grafting of young bone marrow and newborn thymus performed in old mice was effective in restoring the impaired immune functions [5]. The treatment performed in late middle aged was also effective in the restoration of immune functions and the effect was observed even 11 months later [7]. However, when the treatment was performed in early middle aged C3H/MTV female mice, the immunological enhancement appeared to be not satisfactory to delay the onset of mammary adenocarcinoma, as the occurrence of the tumor in C3H/MTV female mice was not altered with no elongation of the mean life span.

Sequential multiple newborn thymus grafting starting at puberty was effective in the retardation of the age-related decline of T cell dependent immune functions starting in the earlier phase of the life. It was shown that sequential grafting of newborn thymus performed every 2 months several times in C3H/MTV mice of the current study and in C57BL/6 in the previous study [4] resulted in the increase of T cells and enhancement of anti-SRBC antibody formation. Moreover, the elongation of the mean life span of certain degree was observed in C3H/MTV and C57BL/6 mice by the sequential grafting of multiple newborn thymus. In the later phase of these experiments, however, additional grafting of newborn thymus appeared to be ineffective in terms of immunological restoration. In the experiment of grafting of multiple old thymuses into adult thymectomized mice, it was revealed that old atrophic thymuses produced factors or cells to suppress the host immune function. In other words, the thymus has two opposing functions, one promoting and the other suppressing immunological functions. The thymic promoting function appears to be predominant in the developing phase and the suppressing one appears to increase with advance of age. Thus, in the experiment of MNTG, multiple thymuses which had been sequentially implanted in the peritoneal cavity could have a suppressive effect on the host immune functions. This might be the most possible explanation why the immunological enhancement was temporarily observed in the first half, but not in the second half of the experiment of MNTG. The retardation of the decline of immune function could delay the onset of disease in aging mice, resulting in elongation of the mean life expectancy to a varying degree, but without change in the maximum life span. When the same treatment started at the middle age, the elongation of mean life expectancy was observed only in a mouse strain in which immunological functions are relatively well maintained until the late stage of the life, but not in a mouse strain in which the immunological functions start to decline earlier [4]. It is interesting to note that both the combined grafting of young bone marrow and newborn thymus, and sequential multiple newborn thymus grafting (MNTG) brought about different effects in an autoimmune prone B/WF1 mice which show an accelerated age-related decline of immune functions. The combined grafting of young bone marrow and newborn thymus resulted in the suppression of

antibody formation, and MNTG aggravated the kidney disease probably by promoting autoantibody formation and resulted in the shortening of the life span. The finding indicates that the composition of T cell subsets in autoimmune prone NZB/WF1 mice are quite different from that of non-autoimmune prone mice. However the treatment with thymosin α-1 decreased the urinary protein, resulting in the elongation of the life expectancy. The beneficial effect of thymosin α-1 in aged mice was also reported in non-autoimmune prone mice [11]. Moreover, Hiramoto reported that thymopoietin [12], a kind of thymic product, was effective in elongating the mean life span of mice. These findings are encouraging for us, as thymic poroducts would be much more convenient for human application and appropriate selective usage of various thymic products in future would be one of most practicable methods to modulate immune responses as well as life expectancy.

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