Mechanisms of Ageing and Development, 28 (1984) 111–121
Elsevier Scientific Publishers Ireland Ltd.

THE EFFECT OF SEQUENTIAL MULTIPLE GRAFTING OF SYNGENEIC NEWBORN THYMUS ON THE IMMUNE FUNCTIONS AND LIFE EXPECTANCY OF AGING MICE

KATSUIKU HIROKAWA and MASANORI UTSUYAMA

Department of Pathology, Tokyo Metropolitan Institute of Gerontology 35-2, Sakaecho, Itabashi-ku, Tokyo (Japan)

(Received July 14th, 1984)
(Revision received September 18th, 1984)

SUMMARY

Enhancement of the immune functions and extension of the mean life expectancy were successfully performed in aging mice by sequential multiple grafting of syngeneic newborn thymus. In the first experiment, 2-month-old female C57BL/6 mice were grafted with either syngeneic newborn thymus or newborn spleen every 2 months, 5 or 6 times. A significant enhancement of T cell dependent immune functions were observed in the group sequentially grafted with newborn thymus, in comparison to that grafted with multiple sequential newborn spleen or with a single newborn thymus and that without a graft. In the second experiment, the same sequential grafting protocol was performed in middle aged mice at monthly interval for 4–5 consecutive months and the immune functions and survival rate were compared between the experimental and control groups. The immune functions were only partially rejuvenated, but an extension of the mean remaining life expectancy was observed in the experimental group (312 ± 38 days) as compared with control (214 ± 42 days), although maximal life-span was the same in both groups (1100 days).

Key words: Sequential multiple thymus grafting; Immune functions; Life expectancy; Aging mice

INTRODUCTION

The thymus starts to involute at around puberty in humans and animals [1,2]. Since the thymic involution precedes or occurs together with the onset of age-related decline of T cell-dependent immune responses, it has been suspected to be partly responsible for the decline. There are two essential thymic functions; i.e. one is production and peri-

0047-6374/84/$03.00
Printed and Published in Ireland

pheralization of T cells, which occurs during late embryonic and early growth phases of life [3,4] and the other is production of thymic hormones, which also peaks at the early growth phase of life, declines rapidly but persists at low level thereafter throughout the life [5,6].

Most of the T cells leaving the neonatal thymus appear to be functionally immature and undergo final functional maturation in the peripheral lymphoid tissues [7,8]. This functional maturation of T cells are believed to occur partly under the influence of thymic hormone. This view is supported by two types of experiments; i.e. one is that adult thymectomy in mice accelerates the decline of T cell dependent immune responses [9,10] and decreases the longevity [11], and the other is that grafting of newborn thymus can substantially restore the activity of T cell immune function of old mice [12]. In the latter case, the restoration of the T cell dependent immune functions is due partly to the recruitment of T cells by the grafted thymus and partly to the hormones secreted by the grafted thymus.

Based on these observations and an earlier observation that the *in situ* host thymus undergoes involution in an orderly pattern independent of that of the graft [13], the present study was carried out to determine the effectiveness of sequential, multiple thymus grafts on T cell-dependent and immunological activities and on the life expectancy starting at 2 and 18 months of age, respectively.

MATERIALS AND METHODS

Mice

Female (C57BL/6NCrj × DBA/2NCrj)F1 (hereafter referred to as BDF1) mice and female C57BL/6NCrj were purchased from Charles River Japan Inc. and used in the present study.

Experimental protocol

In the first experiment, 2-month-old female C57BL/6 mice were intraperitoneally grafted with either one lobe of syngeneic newborn thymus or spleen under anesthesia by Nembutal every 2 months, 5 or 6 times in total. Assessment of the immunological functions were performed at the age of 11 months and 14 months, respectively, and the results were compared with four kinds of control: 3-month-old intact mice, age-matched intact mice, age-matched mice with a single grafting of newborn thymus performed 1 month before the assessment, and age-matched mice which received newborn spleen every 2 months, 5 or 6 times in total, starting at 2 months of age. Each group consisted of 8–10 mice.

In the second experiment, the same thymus grafting protocol was performed in middle aged mice, at monthly intervals for 4–5 consecutive months, and the immune functions and survival rate were compared between the experimental (newborn thymus grafting) and control (newborn spleen grafting) groups.

Mitogenic response

Assays were performed in microplates (Falcon Microtest III, 3072) as previously reported [14]. Briefly, 5×10^5 cells in 0.2 ml of RPMI 1640, supplemented with 5% fetal bovine serum and kanamycin (0.06 mg/ml), were stimulated with optimum doses of either PHA (1 μg; Wellcome Reagent Ltd., England) or LPS (1 μg; *E. Coli*: 0111:B4, Difco Lab., Detroit). The plates were incubated at 37°C in 5% CO_2 in air atmosphere for 66 h, then 0.25 μCi of [^{3}H]thymidine (spec. act. 5.0 Ci/mmol) in 0.005 ml was added, and 2 h later the cells were harvested and processed for beta scintillation counting (LS-250, Beckman).

Anti-SRBC response

Four days after the mice were injected intraperitoneally with 1 ml of 1% SRBC (2×10^8), their spleen cells were assessed for the number of antibody forming cells per spleen by the DPFC assay of Plotz *et al.* [15].

Cell mediated cytolytic T lymphocytes response (CTL)

Five million spleen cells from C57BL/6 (H-2^b) were co-cultured in quadruplicate with an equal number of preirradiated (1500R) spleen cells from C3H (H-2^k) mice in a total volume of 2 ml of RPMI 1640 medium supplemented with 5×10^{-5} M 2-mercaptoethanol, 0.06 mg/ml kanamycin and 10% fetal bovine serum in multiwell plates (24 wells, Corning 25820). The plates were cultured at 37°C in 5% CO_2 in air atmosphere for 5 days, and the cells were harvested and processed for their cytolytic activity according to the method of Cerottini *et al.* [16] as previously described [14].

Mixed lymphocytes reaction (MLR)

One million spleen cells of C57BL/6 mice were co-cultured in microplate (96 wells, Corning 25850) with an equal number of preirradiated (1500R) spleen cells from C3H mice in a total volume of 0.2 ml of the RPMI 1640 medium. The plates were incubated for 70 h, pulsed with 0.25 μCi of [^{3}H]thymidine in 0.005 ml, and cells harvested 2 h later and processed for beta scintillation counting. The stimulation index was obtained by dividing the counts observed in stimulated cultures (experimental) by those obtained in unstimulated cultures (control).

Enumeration of T cells

Spleen cells [10^5] were smeared on a slide glass by Cytospin-II (Shandon, UK) at 500 rev./min for 5 min, fixed with acetone for 2 min, washed with phosphate buffered saline (PBS, pH 7.4) and reacted with biotin conjugated monoclonal antibody to Thy-12 (Beckton Dickinson Monoclonal Center, Inc., CA). The slides were then washed with PBS, reacted with avidin-FITC, washed with PBS and mounted with buffered glycerine (PBS/glycerine = 1:9). The slides were observed under a fluorescent microscope (Zeiss, F.R.G.).

Statistics

Experimental values of immune responses were log-transformed and then the mean value and 1 standard error of the mean were calculated as recommended by Gottlieb [17]. Difference in mean values were assessed by using the Student's *t*-test. The level of significance in the difference of the life expectancy was assessed by the Wilcoxon rank sum test.

RESULTS

Effect of thymus grafting starting at young age

Two-month-old C57BL/6 female mice were intraperitoneally grafted with syngeneic

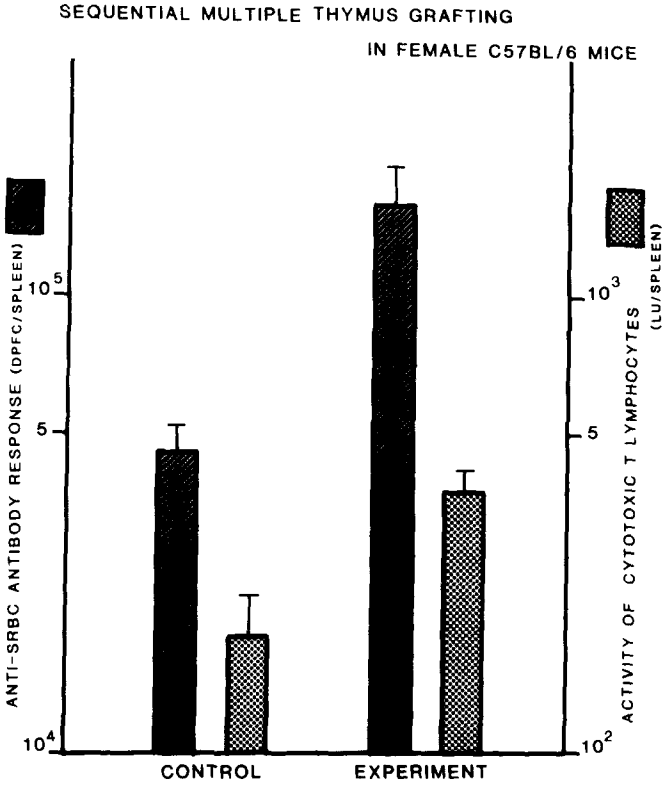

Fig. 1. Anti-SRBC antibody response (DPFC/spleen) and activity of cytolytic T lymphocytes (lytic units/spleen) in female C57BL/6 mice, which had been grafted with either newborn thymus (experimental group) or newborn spleen (control group) every 2 months, 5 times in total. The immunological assessment was performed at the age of 11 months. Levels of anti-SRBC antibody response and activity of cytolytic T lymphocytes in untreated control mice were 573,762 (710,311–463,463) and 568 (699–476) in 3-month-old mice, and 105,398 (176,023–63,758) and 224 (263–190) in 11-month-old mice, respectively (numbers, indicated by geometric mean with range of standard error of the mean in parentheses). Vertical bars, 1 standard error of the mean (S.E.M.). Each group, composed of 6 mice.

newborn thymus every 2 months, and the immunological functions were assessed after 5 or 6 graftings, when mice were 11 or 14 months old, respectively.

At the first interval of immunological assessment (11 months old), two immunological indices were compared (Fig. 1). In the experimental group with sequential graftings of newborn thymus, about a 3.5-fold increase in anti-SRBC antibody response and a 2-fold increase in CTL activity were observed, as compared with the control group receiving newborn spleen graftings. No significant difference was observed in the body weight and spleen weight between these groups.

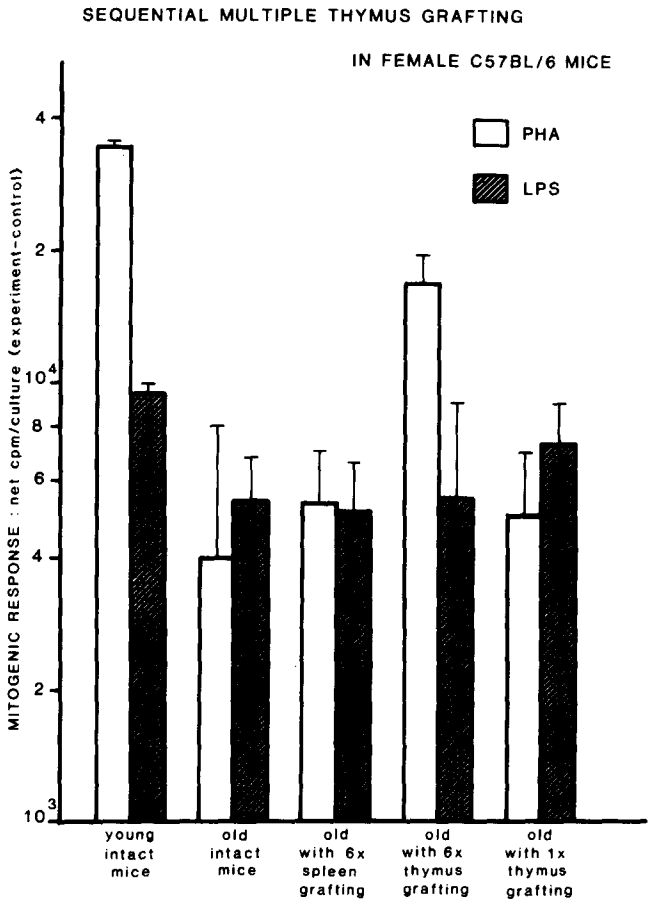

Fig. 2. Mitogenic response to phytohemagglutinin (PHA) and lipopolysaccharide (LPS) in female C57BL/6 mice grafted with newborn thymus every 2 months, 6 times in total starting at 2 months of age (old with 6X thymus graftings). Immunological assessments were performed at 14 months of age. The results were compared with four kinds of control groups: 3-month-old mice (young untreated), 14-month-old age-matched mice (old untreated), 14-month-old mice which had been grafted with newborn spleen every 2 months 6 times in total (old with 6X spleen graftings) and 14-month-old mice which had been grafted with a single newborn thymus, 2 months before the assessment (old with 1X thymus grafting). Vertical bars, 1 S.E.M. Each group, composed of 6 mice.

At the second interval of immunological assessment (14 months old), the experimental group with 6 newborn thymus graftings was compared with 4 kinds of control: young 3-month-old intact mice, age-matched intact mice, age-matched mice with 6 newborn spleen graftings, and age-matched mice with a single newborn thymus grafting performed 1 month before the assessment. As shown in Fig. 2, the PHA response of spleen cells of the experimental group was significantly higher than those of three age-matched controls ($P < 0.01$–0.005) and approached that of young controls. However, no significant difference was observed in the LPS response in all the groups. When assessing the activity of cell-mediated cytolytic T lymphocytes (CTL) the highest response was observed in the experimental group, as compared with young control as well as three age-matched controls ($P < 0.01$–0.001) (Fig. 3). It is important to note that the level of the activity of the experimental group with newborn thymus graftings was significantly higher than the age-matched control with single grafting of newborn thymus, in terms of PHA response (Fig. 2) and CTL activity (Fig. 3).

It is also interesting to note that the weight of *in situ* host thymus in the experimental group was 31.5 ± 1.9 mg, which is not different from that of age-matched intact mice and age-matched mice with 6 graftings of newborn spleen, 28.9 ± 1.2 mg and 30.5 ± 2.5 mg,

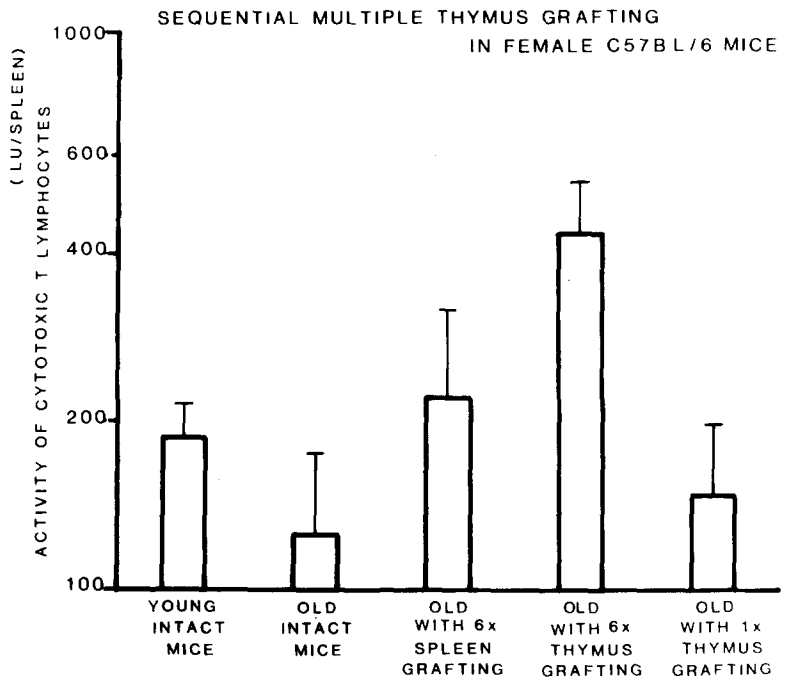

Fig. 3. Activity of cytolytic T lymphocytes in female C57BL/6 mice grafted with newborn thymus every 2 months, 6 times in total, starting at 2 months of age, in comparison to four kinds of controls as described in Fig. 2. Immunological assessment was performed at 14 months of age. Vertical bars, 1 S.E.M. Each group, composed of 6 mice.

TABLE I

THE EFFECT OF SEQUENTIAL MULTIPLE NEWBORN THYMUS GRAFTING ON THE NUMBER OF T CELLS, WEIGHT OF *IN SITU* THYMUS AND BODY WEIGHT

	3 months old young mice	*14 months old control mice*	*14 months old 6× newborn spleen graft*	*14 months old 6× newborn thymus graft*	*14 months old 1× newborn thymus graft*
Percent of T cells in spleen (%)	26.3 ±0.8[a]	23.4 ±2.0	22.2 ±1.4	24.8 ±1.8	21.4 ±1.9
Absolute number of T cells in spleen ($\times 10^7$)	3.96 ±0.40	2.78 ±0.10	2.75 ±0.50	3.28 ±0.33[b]	2.81 ±0.40
Total cell counts in spleen ($\times 10^7$)	15.18 ±1.79	13.07 ±1.15	11.96 ±1.51	13.56 ±1.26	11.69 ±0.66
Wet weight of *in situ* thymus (mg)	66.6 ±4.8	28.9 ±1.2	30.5 ±2.5	31.5 ±1.9	27.3 ±1.4
Body weight (g)	19.5 ±0.4	25.5 ±1.2	28.3 ±0.9	27.9 ±0.7	29.3 ±1.0

[a] Values indicate the mean ± 1 standard error.

[b] Absolute number of T cells in spleen in the group with 6× newborn thymus graft is significantly higher ($P < 0.01$) than in other age-matched control groups.

respectively (Table I). No significant difference was observed in body weight and total splenic cell counts among these age-matched groups, regardless of the treatment. The percentage and absolute number of T cells in spleen decreased with advance of age in C57BL/6 mice. However, this decline could be restored to the level of young by sequential multiple grafting of newborn thymus starting at 2 months of age, but not by sequential grafting of newborn spleens or by a single grafting of newborn thymus (Table I).

Effect of thymus grafting starting at middle age

In this series of experiments, sequential multiple grafting of either newborn thymus (experimental group) or spleen (control group) were performed in middle aged C57BL/6 and BDF1 female mice.

In C57BL/6 mice, the sequential graftings were performed every month, 4 times in total, starting at 17 months and ending at 20 months of age, and the immunological functions were assessed 1 month after the last treatment. As shown in Fig. 4, a significant increase of immune response was observed only in PHA response showing about 30% of young adult level, not in anti-SRBC antibody response and mixed lymphocyte reactions (MLR).

In BDF1 mice, the sequential graftings were performed every month, 5 times in total, starting at 18 months and ending at 22 months of age, and the survival rate was observed as shown in Fig. 5. The mean life expectancy from age of 18 months of the experimental

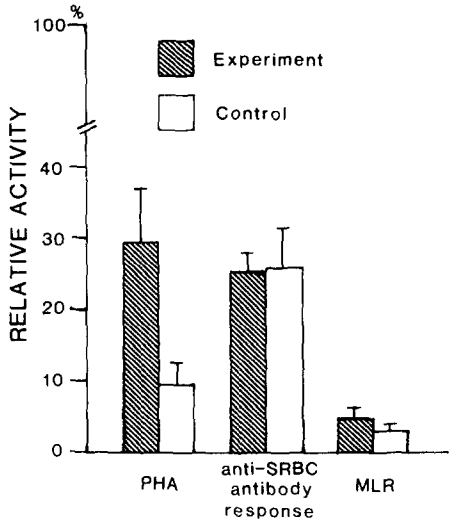

Fig. 4. Effect of sequential multiple thymus grafting started at middle age on the immune functions. 17-month-old female C57BL/6 mice were grafted with either newborn thymus (experimental group, hatched columns) or newborn spleen (open columns) every month for 4 consecutive months. The immunological assessment was performed 1 month after the last treatment. The relative activity was expressed as percentage of level of 3-month-old female mice. PHA, mitogenic response to phytohemagglutinin. MLR, mixed lymphocytes reaction. Vertical bars, 1 S.E.M. Each group, composed of 6 mice.

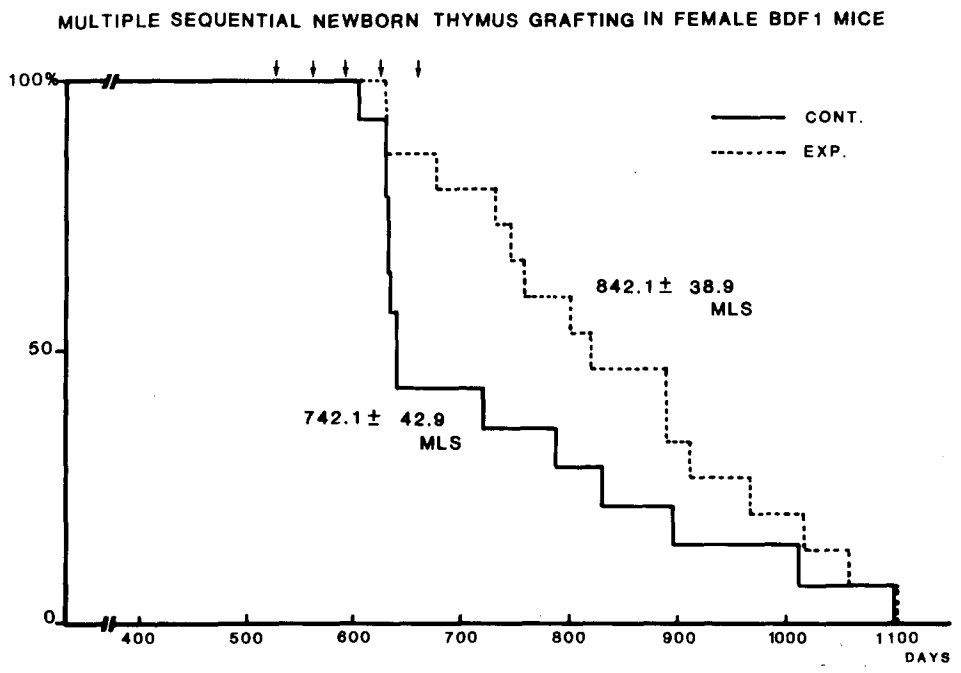

Fig. 5. Comparison of survival rate between experimental (broken line) and control group (continous line). Either newborn thymus (experimental) or newborn spleen (control) was grafted every month, starting at 18 months of age (arrows), 5 times in total. Each group, composed of 15 mice. The mean life expectancy starting at age 18 months of the experimental and control groups were 313 ± 38 and 214 ± 42 days, respectively ($P = 0.08$).

group was 312 ± 38 days, which is slightly longer than that of control group, 214 ± 42 days ($P = 0.08$). However, there was only a marginal difference in the maximal life span (about 1100 days) between two groups.

DISCUSSION

In normal mice, the thymus grows rapidly after birth and starts to involute at 4–6 weeks of age [2]. Associated with this involution, the thymic activity to premote T cell differentiation also starts to decline at around puberty [2,14]. Therefore, the maintenance of thymic function at the level of newborn and young adult level would appear to be essential in preventing the age-related decline of T cell-dependent immune functions, and the implantation of newborn thymus was revealed to be the most efficient way to do this in mice [2,14].

In implanting thymus gland into mice, Metcalf [13] initially found that the greater number of thymus glands grafted, the larger was the total thymic mass. Multiple thymus grafting was then shown to be effective in restoring the immune functions of neonatally thymectomized mice [18,19]. However, once immune functions reached a peak plateau

level, additional thymus graft had no effect on enhancing the immune functions [20], although the number of lymphocytes increased. Since all these thymus grafts undergo involution at a fixed rate regardless of their number, e.g. 6 weeks in adult mice, it would seem that sequential thymus grafting protocol would be very effective in preventing the age-related decline of immune functions.

The present study, revealed that the sequential multiple thymus grafting protocol, when started at young age, is indeed very effective in significantly slowing the decline of T cell-dependent immune functions, as judged by the anti-SRBC, CTL and PHA responses. In fact, the level of CTL activity was enhanced over the level of young adult mice. These upward modulating effects were functionally associated with a significant increase in the total number of T cells in the spleen. Such an increase of T cells could be ascribed partly to the accumulation of T cells produced by each thymus grafts and partly to enhanced proliferation of T cells in the peripheral lymphoid organs under the influence of thymic hormones maintained at the level of young by sequential thymus grafting. The fact that weight of *in situ* thymus was not affected by the sequential multiple thymus grafting is consistent with the view that the growth and involution of the thymus is an autonomous behaviour [13]. But when the same grafting protocol is started at middle age, it is only partially effective in enhancing the decreased PHA response and totally ineffective in restoring other immune functions such as anti-SRBC antibody response and MLR. The findings are consistent with our previous report [12] that the combined transplantation of both young bone marrow cells and newborn thymus was obligatory for the restoration of immune functions of old mice.

However, it is encouraging that the sequential multiple thymus grafting starting at middle age resulted in an extension of the mean remaining life expectancy. The change in the maximal life span, however, was marginal. These results support the notion that the maximal life span of these aging mice could be extended by starting the sequential thymus grafting at an earlier age and extending its period beyond 5 months as done here.

ACKNOWLEDGEMENTS

The authors express their appreciation to Dr. T. Makinodan (director of GRECC, VA, Wadsworth Medical Center, LA) for his kind advice in the preparation of the manuscript.

REFERENCES

1 E. Boyd, The weight of the thymus gland in health and in disease. *Am. J. Dis. Child., 43* (1932) 1162–1214.
2 K. Hirokawa and T. Makinodan, Thymic involution: Effect on T cell differentiation. *J. Immunol., 114* (1975) 1659–1664.
3 J.F.A.P. Miller, Immunological function of the thymus. *Lancet, II* (1961) 748–749.
4 R. Scollay, E.C. Butcher and I.L. Weissman, Thymus cell migration. Quantitative aspects of cellular traffic from the thymus to the periphery in mice. *Eur. J. Immunol., 10* (1980) 210–218.
5 J.E. McClure, N. Lameris, D.W. Wara and A.L. Goldstein, Immunochemical studies on thymosin: Radioimmunoassay of thymosin α_1, *J. Immunol., 128* (1982) 368–375.

6 K. Hirokawa, J.E. McClure and A.L. Goldstein, Age-related change in localization of thymosin in the human thymus. *Thymus, 4* (1982) 19–29.
7 O. Stutman, Intrathymic and extrathymic T cell maturation. *Immunol. Rev., 42* (1978) 139–184.
8 P.-F. Piguet, C. Irle, E. Kollatte and P. Vassalli, Post-thymic T lymphocytes maturation during ontogenesis. *J. Exp. Med., 154* (1981) 581–593.
9 J.F.A.P. Miller, Effect of thymectomy in adult mice on immunological responsiveness. *Nature, 208* (1965) 1337–1338.
10 K. Hirokawa and Y. Hayashi, Effect of adult thymectomy on immune potentials, endocrine organs and tumor incidence in long-lived mice. *Adv. Exp. Med. Biol., 129* (1980) 243–247.
11 H.F. Jeejeebhoy, Decreased longevity of mice following thymectomy in adult life, *Transplantation, 12* (1971) 525–526.
12 K. Hirokawa, J.W. Albright and T. Makinodan, Restoration of impaired immune functions in aging animals. I. Effect of syngeneic thymus and bone marrow grafts. *Clin. Immunol. Immunopathol., 5* (1976) 371–376.
13 D. Metcalf, Multiple thymus grafts in aged mice. *Nature, 208* (1965) 87–88.
14 K. Hirokawa, K. Sato and T. Makinodan, Influence of age of thymic grafts on the differentiation of T cells in nude mice. *Clin. Immunol. Immunopathol., 24* (1982) 251–262.
15 P.H. Plotz, N. Talal and R. Asofsky, Assignment of direct and facilitated hemolytic plaques in mice to specific immunoglobulin classes. *J. Immunol., 100* (1968) 744–751.
16 J.C. Cerottini, H.D. Egner, H.R. MacDonald and K.T. Brunner, Generation of cytolytic T lymphocytes in vitro. I. Response of normal and immune mouse spleen cells mixed leukocytes cultures. *J. Exp. Med., 140* (1976) 703–717.
17 C.F. Gottlieb, Application of transformation to normalize the distribution of plaque-forming cells. *J. Immunol.*, 113 (1974) 51–57.
18 R.T. Schaller, Jr., J. Schaller and J.K. Stevensen, Reversal of wasting syndrome in thymectomized mice by multiple syngeneic or allogeneic thymus grafts. *J. Natl. Cancer Inst., 38* (1967) 287–304.
19 O. Stutman, Reversal of post-thymectomy wasting disease in mice by multiple thymus graft. *J. Immunol., 98* (1967) 79–87.
20 A. Kunii, J. Furth and L. Berwick, Studies on restoration of sensitivity of thymectomized rats to viral leukemia. *Cancer Res., 26* (1966) 48–59.