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Differential Rate of Age-Related Decline in Immune Functions in Genetically Defined Mice with Different Tumor Incidence and Life Span

Katsuiku Hirokawa, Masanori Utsuyama, Hiroshi Goto, Kazunao Kuramoto

Department of Pathology, Tokyo Metropolitan Institute of Gerontology, Sakaecho, Itabashi-ku, Tokyo, Japan

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Abstract. Immunological studies were performed in aging C57BL/6 and BDF_1 mice. In C57BL/6 mice the incidence of malignant tumor was high and their mean life span was 665 days in males and 649 days in females. In BDF_1 mice, the incidence of malignant tumors was significantly lower, and their mean life span was 954 days in males and 846 days in females. Immunologically, either the onset of decline occurred earlier, the rate of the age-related decline in activity was more rapid, and/or the magnitude of decline was greater in the shorter-lived C57BL/6 than in the longer-lived BDF_1 mice. The close relationship between (a) the susceptibility especially to malignant lymphoma; (b) the onset, rate and magnitude of age-related decline in T-cell-dependent immunologic activities, and (c) the life span would suggest that T-cell immunity may be playing an important role in the resistance against life-shortening malignant lymphoma in C57BL/6 mice.

Introduction

The immune system has been a focus of intense gerontological research, because it shows striking alterations during aging. Moreover, altered immune functions can predispose individuals to various deleterious diseases (cancer, infection and cardiovascular and renal disease) [7, 13]. If these changes were causally related, individuals showing a slower rate of age-related decline in immune functions should be less vulnerable to age-related diseases and therefore survive longer than individuals with a faster rate of age-related decline in immune functions. To resolve this issue, a comprehensive immunological examination of an inbred strain and its hybrid, which differed in their susceptibility to tumor and in life span, was performed.

Materials and Methods

Mice

4-week old C57BL/6 (C57BL/6NCrj) and BDF_1 (C57BL/6NCrj × DBA/2NCrj) F_1 mice were purchased from Charles River Japan Inc., and reared and

raised in our specific pathogen-free mouse colony. 3, 6, 12, 18 and 24-month-old C57BL/6 and BDF_1 mice of both sexes in groups of 20–30 were randomly selected and sacrificed for the present study, totaling about 500 mice. At sacrifice, their spleens were aseptically removed and a fraction was assessed for immunological activities and another faction fixed with 10% formalin for histopathological examination. Immunological data obtained from those mice with cancerous spleens were excluded from statistical analyses.

Mitogenic Response

Assays were performed in microplates (Falcon Microtest III, 3072) as previously reported [5]. 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 phytohemagglutinin (PHA: 1 μg; Wellcome Reagent Ltd., England), concanavalin A (Con A: 1 μg; Sigma Chemical Co., St. Louis, Mo.) or *Escherichia coli* lipopolysaccharide (LPS: 1 μg; *E. coli*:0111:B4, Difco Lab., Detroit, Ill.). The plates were incubated at 37 °C in 5% CO_2 in air atmosphere for 66 h, then 0.25 μCi of H^3-thymidine (s.a. 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

4 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 direct plaque forming cells (DPFC) assay of *Plotz* et al. [11].

Cell-Mediated Cytolytic T Lymphocyte (CTL) Response

5 million spleen cells from C57BL/6 ($H\text{-}2^b$) or BDF_1 ($H\text{-}2^{bd}$) were co-cultured in quadruplicate with an equal number of preirradiated (1,500 R) spleen cells from C3H (C3H/NCrj) ($H\text{-}2^k$) 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 calf 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. [1] as previously described [5].

Mixed Lymphocyte Culture (MLC)

1 million spleen cells of mice were co-cultured in microplate (96 wells, Corning 25850) with an equal number of preirradiated (1,500 R) 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 3H-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).

Natural Killer (NK) Activity

A fixed number of ^{51}Cr-labeled YAC target cells (4×10^4) was mixed with either 1, 2, or 4×10^6 spleen cells in a total volume of 0.2 ml in microplate with round bottomed wells. The plates were incubated for 5 h, and the percent specific ^{51}Cr release was estimated by using the method employed in assessing cytolytic T-cell activity.

Autoantibody against Bromeline-Treated Mouse RBC

1 million spleen cells in 0.2 ml of RPMI 1640 supplemented with 10% fetal bovine serum, 5×10^{-5} *M* 2-mercaptoethanol and 0.06 mg/ml kanamycin were stimulated with an optimum dose of LPS (1 μg) in microplates [9]. The plates were incubated at 37 °C in 5% CO_2 in air atmosphere for 72 h, when the cells were harvested and assessed for the number of antibody-forming cells against bromelain-treated syngeneic RBC, using the same method to assess antibody-forming cells against SRBC. Bromelain-treated mouse RBC were prepared as follows; 0.5 ml of washed and packed RBC were suspended in 1.5 ml of bromelain solution (50 mg/ml in RPMI 1640 medium) (Nakarai Chem. Ltd., Japan), incubated at 37 °C for 40 min, and washed 3 times in RPMI 1640 medium.

Interleukin-2 (IL-2) Production

0.5 million cells were stimulated with 1 μg of Con A and 1 day later the supernatants were harvested and filtered through an 0.22-μm Millex filter. A volume of 100 μl of the supernatants was mixed with 100 μl of IL-2 dependent CTLL-1 cells (5×10^3) in triplicate and incubated at 37 °C for 24 h in a CO_2 incubator; 0.25 μCi of H^3-thymidine in 0.005 μl was then added, and the cells were harvested 4 h later and processed for

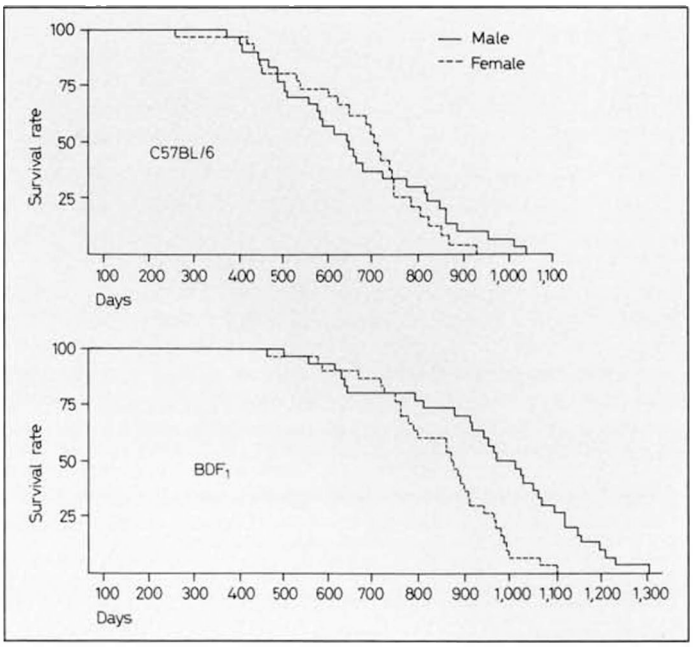

Fig. 1. Survival patterns of aging C57BL/6 and BDF_1 mice. Sample size, 30 per group.

scintillation counting [2]. The dilution of the supernatant with the highest cpm was selected to express the activity of each individual mouse, as our preliminary analysis of the data indicated that the cpm index was comparable to the relative unit index.

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* [3]. Differences in mean values were assessed by using the Student's t test.

Results

Lymphoma Incidence and Life Span

The mean life span of C57BL/6 was 665 ± 188 days in males and 649 ± 162 days in females, and that of BDF_1 was 954 ± 215 days in males and 846 ± 147 days in females (fig. 1). A high incidence of malignant lymphoma was observed in C57BL/6 mice dying naturally; by 24 months of age, 55% of males and 60% of females died from malignant lymphoma, with tumor onset beginning at around 12 months of age. Other tumors were very few and almost negligible. In male BDF_1 mice, 5% died from malignant lymphoma and 5% from hepatoma by 24 months of age. In females, 7% died from malignant lymphoma and 8% from uterine sarcoma by 24 months of age. The onset of tumors began at around 18 months of age in these longer-lived hybrid stock mice (fig. 2).

Mitogenic Responses

The PHA response declined with age in both C57BL/6 and BDF_1 mice (fig. 3), but the onset and rate of decline differed between them. In the shorter-lived C57BL/6 mice, the decline began at 12 weeks of age, and was dramatic and exponential in pattern (i.e. a

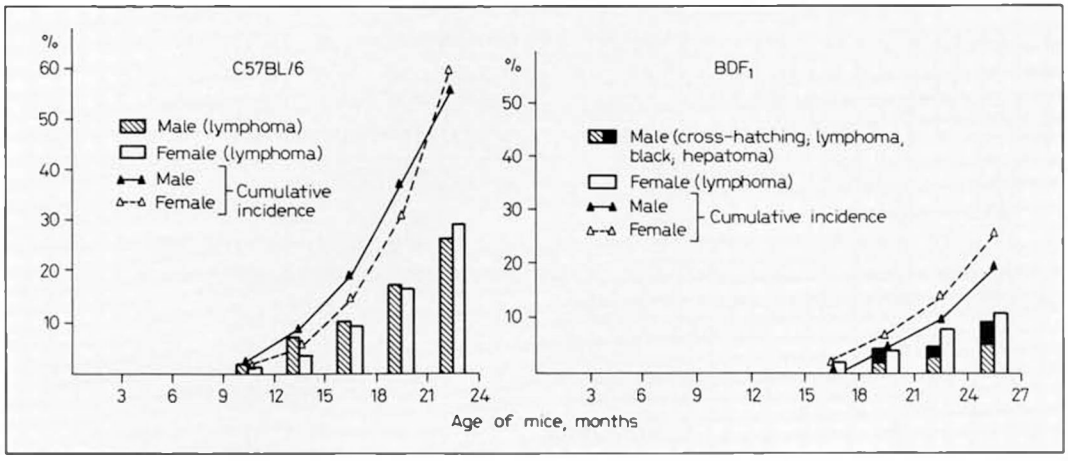

Fig. 2. Cumulative incidence of malignant tumors in C57BL/6 and BDF_1 mice.

straight line decline in a semilog plot as shown in figure 3). In the longer-lived BDF_1 mice, the onset of decline occurred later at around 53 weeks of age, and the rate of decline appeared faster. It is interesting to note that the individual variation of PHA response increased with age in both test populations of mice. No significant difference could be detected between sexes of either test population of mice, and therefore the data on male mice are not presented.

The pattern of age-related response to Con A was comparable to that of PHA, and no change in the LPS-induced mitogenic response could be detected with age in both test populations of mice, and therefore the data are not presented.

MLC Response

As with the PHA response, the MLC response also declined with age in both the C57BL/6 and BDF_1 mice (fig. 4). The decline in C57BL/6 mice began at around 12 weeks of age, and the rate of decline was rapid until 52 weeks of age, when it leveled off. In contrast, the BDF_1 mice showed only a slight age-related decline starting at around 38 weeks of age. No sex-related differences could be detected with either C57BL/6 or BDF_1 mice.

Anti-Sheep RBC Response

Age-related decline was observed in the T-cell-dependent antibody response against SRBC in both the C57BL/6 and BDF_1 mice, but their rate and pattern of decline differed as with the PHA and MLC responses (fig. 5). Thus, a dramatic progressive decline was observed beginning at the peak level at 12 weeks of age in C57BL/6 mice, and the magnitude of decline between 12 and 104 weeks was 100-fold in both sexes. In contrast, the onset of decline in BDF_1 mice occurred after 52 weeks of age, and the magnitude of decline between the peak level and that at 104 weeks of age was 2-fold in the male and 4-fold in the female. As with the PHA and MLC responses, an increase in individual variation with age was also prominent in both populations of test mice.

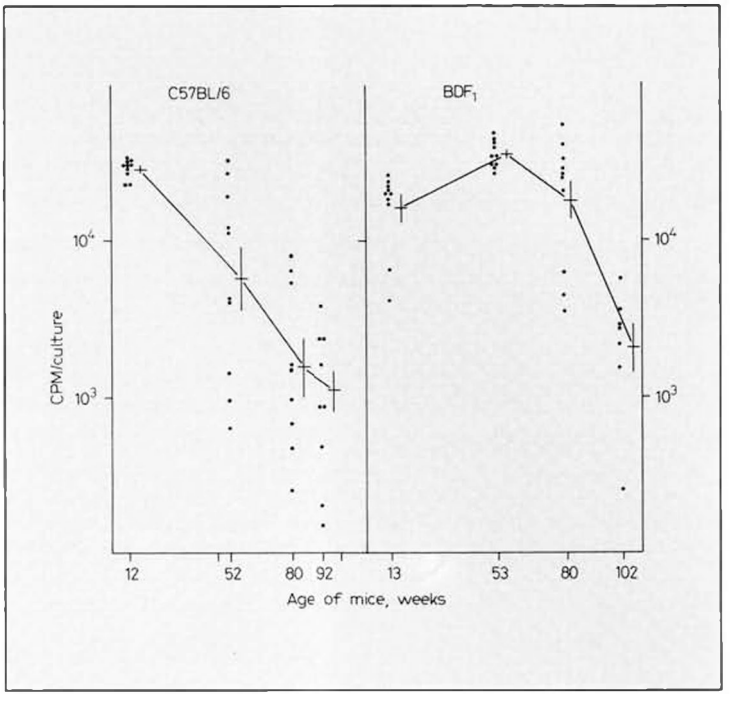

Fig. 3. PHA response of splenic lymphocytes of female C57BL/6 and BDF_1 mice. Solid circle, activity of individual mouse; horizontal bar, mean; vertical bars, 1 SEM.

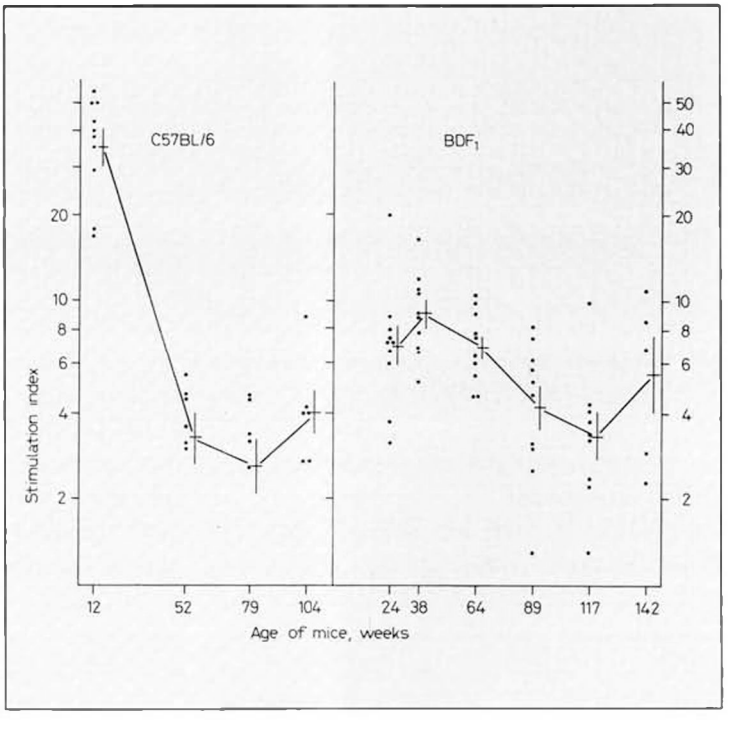

Fig. 4. MLC response by splenic lymphocytes of male C57BL/6 and female BDF_1 mice. The activity was expressed by stimulation index. The unstimulated control had a background of 553 ± 26 cpm/culture in C57BL/6 and 1,128 ± 76 cpm/culture in BDF_1. Solid circle, activity of individual mouse; horizontal bar, mean; vertical bar, 1 SEM.

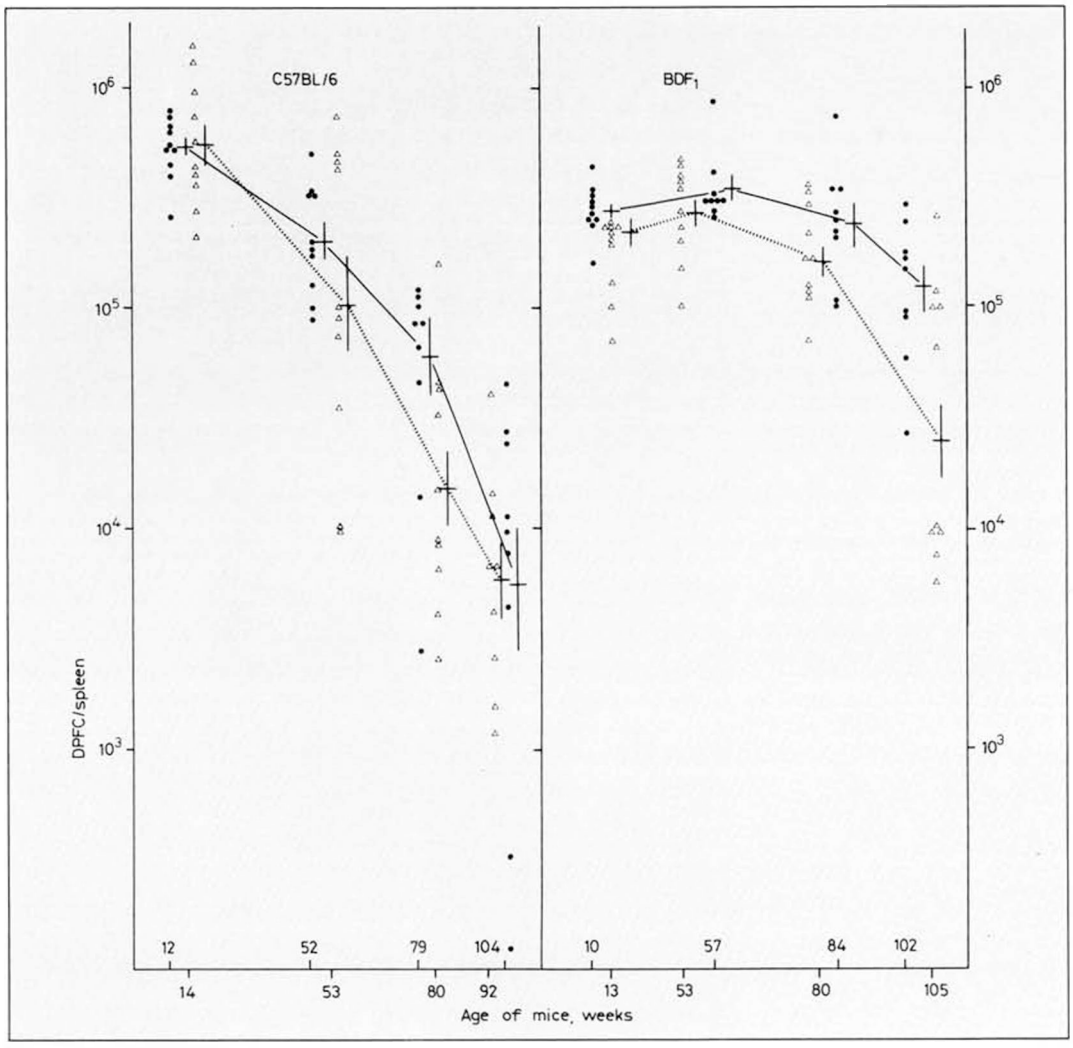

Fig. 5. Anti-sheep RBC response by splenic lymphocytes of C57BL/6 and BDF_1 mice. Solid circle, male; open triangle, female; horizontal bar, mean; vertical bar, 1 SEM.

CTL Response

In C57BL/6 mice, the CTL response declined linearly in a semilogarithmic plot, beginning at 14 weeks of age in the female and 52 weeks in the male (fig. 6). A linear decline in CTL response could also be detected in BDF_1 mice, starting at around 24 weeks of age. With both sexes, the decline continued until about 90 weeks of age when it leveled off in the female and increased in the male. Interestingly, there were a number of male and female BDF_1 mice at 102 weeks and older whose CTL response was higher than the mean response of young mice.

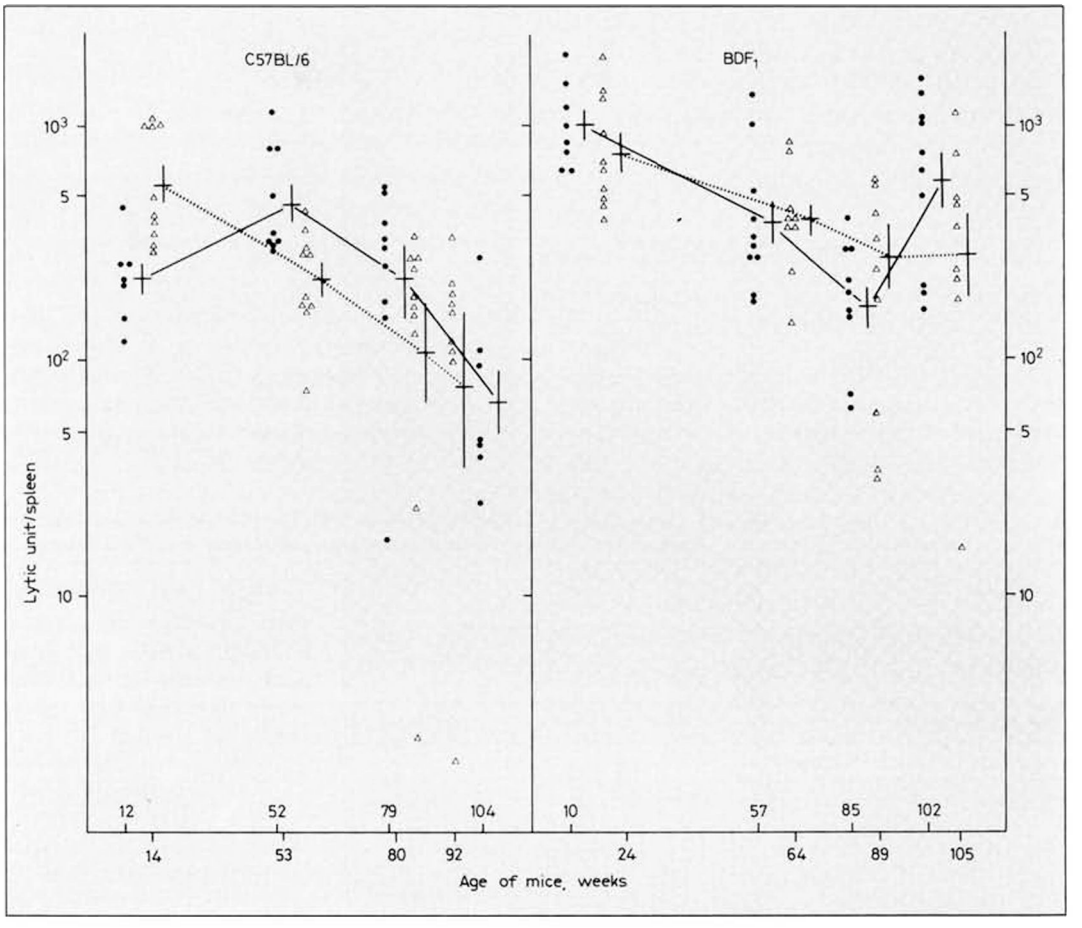

Fig. 6. CTL response by splenic lymphocytes of C57BL/6 and BDF_1 mice. Solid circle, male; open triangle, female; horizontal bar, mean; vertical bar, 1 SEM.

NK Activity

As shown in figure 7, the results indicate that the NK activity in both the C57BL/6 and BDF_1 mice also declined linearly with age, starting at around 12 weeks of age. Although the sampling points were limited in the case of BDF_1 mice, the results would suggest that if a difference in the rate of decline exists between them, it would be minimal. Unlike other immunological indices assessed, a large individual variation in NK activity was observed independent of their age in both populations of test mice.

LPS-Induced Autoantibody Response

Our sampling of C57BL/6 mice, which did not extend beyond 92 weeks of age, suggests that a slight increase may occur between 14 and 80 weeks of age (fig. 8). However, an abrupt decrease was apparent at 92 weeks of age. With BDF_1 mice, a slight decrease was detected between the peak level at 38 weeks

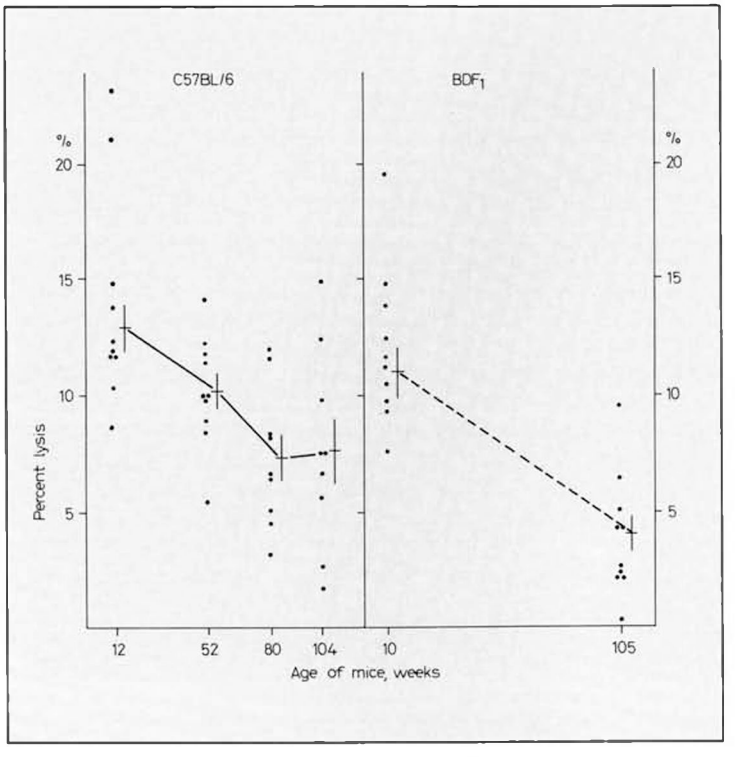

Fig. 7. NK activity by splenic lymphocytes of male C57BL/6 and BDF_1 mice. Solid circle, activity of individual mouse; horizontal bar, mean; vertical bar, 1 SEM.

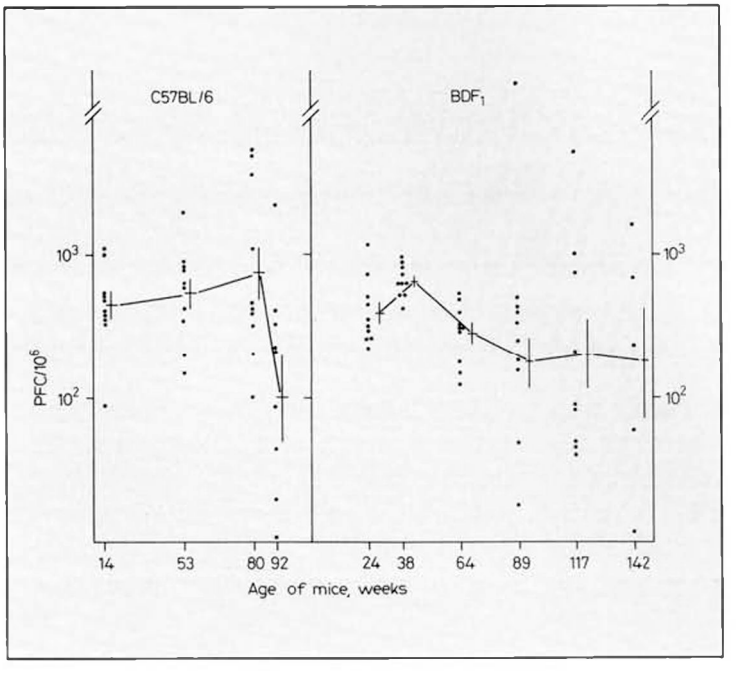

Fig. 8. LPS-induced autoantibody production by splenic lymphocytes of female C57BL/6 and BDF_1 mice. Solid circle, activity of individual mouse; horizontal bar, mean; vertical bar, 1 SEM.

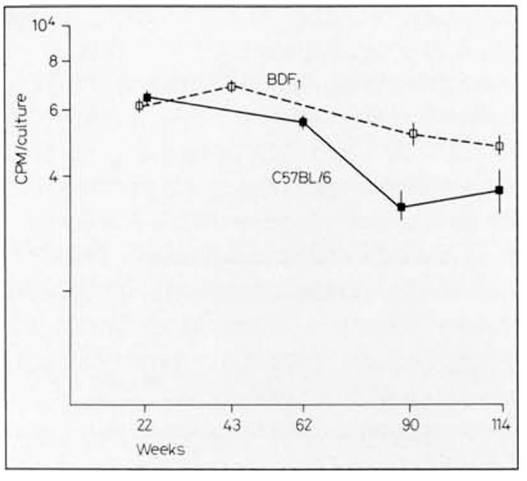

Fig. 9. IL-2 production by splenic lymphocytes of female C57BL/6 and BDF_1 mice. Vertical bar, 1 SEM.

of age and 89 weeks of age, after which the response leveled off. However, as with the CTL response, there were several mice of 80 weeks and older in both populations of test mice whose response levels were higher than the highest observed in young mice.

IL-2 Production

A moderate age-related decline in IL-2 production was observed in both the C57BL/6 and BDF_1 mice, but the rate of decline appeared to be slightly more pronounced in the shorter-lived C57BL/6 mice than in the longer-lived BDF_1 mice (fig. 9).

Discussion

About 5 years ago, *Walford* and co-workers [8, 12] demonstrated that the major histocompatibility complex (MHC) played an important role in immunosenescence and life span of aging mice. Since then, there has been only a minimal effort devoted to the resolution of the relationship between immunosenescence, disease susceptibility and life span. We have therefore undertaken a comprehensive study investigating the immunological parameters and disease patterns of two closely related, genetically defined mice with different life spans. The animals we selected for our initial study were the shorter lived C57BL/6 mice (mean life span, 665 ± 188 days for males and 649 ± 162 days for females) and the longer-lived BDF_1 mice (mean life span, 954 ± 215 days for males and 846 ± 147 days for females). The results reported here are focused on age-related changes in T-cell-dependent immunological activities and susceptibility to malignant tumors, especially malignant lymphoma.

We found that all the T-cell-dependent splenic immunological activities which were assessed showed a decline with age. They were PHA, Con A, MLC, CTL and anti-sheep RBC responses and IL-2 production. Moreover, a wide variation in individual responses with age was noted. Thus with some individual old mice, the level of activity was comparable to or even higher than the mean level of activity of young mice. One implication of this latter observation is the need to use a sufficiently large sample size in assessing age-related T-cell-dependent immunological activities. These findings confirm the former studies demonstrating the vulnerability of T-cell-dependent immunological activities to aging. What is more important, however, are the results demonstrating for the first time that in general either the onset of decline occurred earlier, the rate of decline was faster, and/or the magnitude of decline was more pronounced in the shorter-lived C57BL/6 mice than in the longer-lived BDF_1

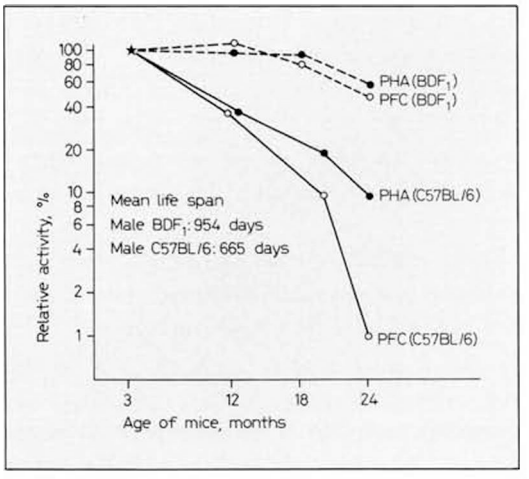

Fig. 10. Comparison of the rate of age-related decline of immune responses between C57BL/6 (– – –) and BDF_1 (——) male mice. ● = PHA response; ○ = anti-SRBC antibody response (PFC).

mice (fig. 10). It is apparent that had we employed the more commonly used sampling method of assessing only young and old mice, we would not have been able to detect two of the three types of age-related changes. Furthermore, the magnitude of age-related changes could have been underestimated had we employed such a method, as the results show that the age of peak activity differed within and between the two test populations of mice, depending upon the specific immunologic index. Thus, these findings underscore the importance of assessing multiple points along the vector in determining age-related changes in immunologic activities.

B-cell activities, as judged by LPS-induced mitogenic response (data not presented) and T-cell-independent antibody response [unpublished], showed no age-related change. As with age-related T-cell-dependent immunologic activities, these findings are consistent with the former studies [7, 13]. However, in contrast to the findings reported by *Naor* et al. [10], we did not detect an age-related increase in the frequency of LPS-induced autoantibody forming cells in both the C57BL/6 and BDF_1 mice, as judged by the number of antibody-forming cells against bromelain-treated mouse RBC. The discrepancy could be due to differences in the selection of the mouse, sample size, and/or frequency of sampling along the age vector (i.e. young and old comparison versus multiple age group comparison). It should be noted, however, that although the mean number of LPS-induced autoantibody-forming cells decreased with age in our study, a substantial number of individual old mice possessed numbers higher than the highest among the young mice. Thus, these results again underscore the importance of the need to use a sufficiently large number of mice for age-related studies. The NK activity declined with age in both test mice, as in the case of T-cell-dependent immunological activities. This confirms the observations of earlier investigators [4, 6]. Since no significant difference was observed in the rate of decline between C57BL/6 and BDF_1, NK activity may not be directly related to tumor susceptibility in these mice.

Finally, a close correlation was established between immunosenescence, as reflected by the age-related decline in T-cell-dependent immunological activities, and susceptibility to malignant tumors, especially malignant lymphoma. Thus, the results revealed that the shorter-lived C57BL/6 mice with an immune system that is more vulnerable to aging than the BDF_1 mice were more susceptible to malignant tumors, as judged by the age of onset of susceptibility (C57BL/6, 12 months; BDF_1, 18 months), and the incidence (C57BL/6, 55% in males and 60% in fe-

males; BDF_1, 10% in males and 15% in females, by 24 months of age).

These studied therefore establish that a relationship exists between: (a) T-cell-dependent immunological activities; (b) susceptibility to malignant tumors, especially lymphoma, and (c) life span. Further studies are therefore needed to establish whether these parameters are causally related.

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Dr. Katsuiku Hirokawa,
Department of Pathology,
Tokyo Metropolitan Institute of Gerontology,
35–2, Sakaecho, Itabashi-ku,
Tokyo-173 (Japan)