Immunological Function of Food-restricted Germfree and Specific Pathogen-free Mice

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The effects of food restriction on immune function was investigated in germfree (GF) and specific pathogen-free (SPF) mice. They were maintained from five weeks of age under either full-fed or food-restricted conditions to 4.5 grams per day (equivalent to approximately 80% of full-fed intake) of a commercial diet. Longest survival rate was attained in food-restricted SPF mice followed by food-restricted GF, full-fed GF, and full-fed SPF animals. Food-restricted GF mice showed shorter survival rate than their SPF counterparts. This result suggests that food restriction may be just as effective as GF status for extending life span. Immune function declined significantly with age in full-fed groups of GF and SPF mice. In both food-restricted GF and SPF mice, mitogenic response to concanavalin A or lipopolysaccharide and antibody response to sheep red blood cells were lower early in life and became higher later in life as compared with full-fed mice. Hence, the maintenance of effective immunological function until old age may be the reason for food-restricted groups to live slightly longer than full-fed groups. — KEY WORDS: food restriction, germfree mice, life span, mitogenic response, plaque-forming cells response

We are currently conducting a long-term study on aging in germfree (GF) mice. GF animals are believed to be suitable models for aging research because mortality is not affected by microbial infection. Gorden et al. [3] reported that GF mice outlived conventional (CV) mice. Pollard and Wostmann [15] showed that full-fed GF Lobund-Wistar (L-W) rats outlived their full-fed CV counterparts. Hence, it was considered that the longer life span of the GF animals may be due to unique characteristics of the GF state.

It is a well-known fact that food restriction started either early in life or in adult life markedly increases both mean and maximum life span [1, 9, 17, 27, 30], reduces incidence and delays onset of cancer and other late-life diseases [2, 10, 18, 22, 27, 30] in rodents. From these results, it appears food restriction affects basic mechanisms of the aging process common to these rodents.

It is now well recognized that the activity of the immune system declines with advancing age [11]. It is also known that food restriction produces striking effects on immunological function [4, 5, 8, 29]. Old rodents subjected to restricted diets since weaning show stronger immunological responses than age-matched full-fed controls [26, 28].

Very few reports describe the aging processes in GF animals subjected to food restriction from weaning through their usual or extended life spans [19, 20]. Not surprisingly, there have been no studies on the relationships between the effects of GF status, food restriction and immune functions in aging animals. The present study was designed to examine the effects of food restriction on the immune responses of aging GF mice.

Materials and Methods

Mice and diets: Four-week-old GF and specific pathogen-free (SPF) male ICR mice
were purchased from CLEA Japan Inc., Tokyo, Japan. GF mice, reproduced and maintained in plastic isolator systems, were free of detectable microflora. The SPF mice were maintained in air-conditioned standard animal room quarters. Full-fed mice (GF-F and SPF-F) were housed three to a plastic cage while food-restricted mice (GF-R and SPF-R) were housed individually in small steel cages. All animals were provided with laboratory diet (CL-2, CLEA Japan), tap water, bedding and cages, sterilized by steam. Intake was restricted to 4.5 grams of food per day beginning at 5 weeks of age in the food-restricted animals, equivalent to approximately 80% of full-fed (ad libitum) intake. Representative mice were sacrificed at weeks 25, 40, 70, 90, 105, and 140 of age to permit studies on immunological function.

Preparation of spleen cells: Spleens were aseptically removed from the mice. They were gently teased between the frosted ends of two sterile microscope slides into cold RPMI 1640 medium (GIBCO, Grand Island, N. Y., USA), supplemented with antibiotics (GIBCO), and passed through sterile stainless steel mesh. The cells were washed twice in the same medium. Viable cells were counted by trypan blue exclusion.

Hemolytic plaque assay: The cells were adjusted to 2.5-5.0 x 10⁶ cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum (Bockneck, Ontario, Canada), 25 mM HEPES buffer (GIBCO), 200 mM L-glutamine (GIBCO), antibiotics, and 5 x 10⁻⁵ M dimercaptoethanol (Wako Pure Chemical Industries Ltd., Osaka, Japan). The cultures in triplicate were incubated with 8 x 10⁶ sheep erythrocytes (SRBC, Nippon Bio-Test Laboratories Inc., Tokyo, Japan) in tissue culture multi-well plates (Flow Laboratories Inc., Mclean, VA, USA) at 37°C in a humidified atmosphere with 5% CO₂ in air for 5 days. Before assay, the triplicate cultures in each group were pooled. The test for the in vitro plaque-forming cells (PFC) response to SRBC were enumerated using the modification of Mishell and Dutton [12].

Lymphocyte response to mitogens: The proliferative response of lymphocytes was tested by the modified method of Strong et al. [21]. Briefly, spleen cells were dispensed in round-bottomed microtiter wells (Corning Glass Works, N. Y., USA) at 1 x 10⁶ cells per well in medium containing mitogen or in medium alone. The cells were incubated for 48 hr at 37°C in a humidified atmosphere with 5% CO₂ in air. Mitogens used in this study were 1 or 5 µg/ml concanavalin A (Con A, SIGMA Chemical Co., St. Louis, MO, USA) and 1 or 5 µg/ml lipopolysaccharide (LPS, E. coli 0127: B8. DIFCO Laboratories Inc., Detroit, MI, USA). One µCi of [6-³H] thymidine (2 Ci/mM, Amersham Japan, Tokyo, Japan) in 10 µl of RPMI 1640 medium supplemented with antibiotics was added to each well 4 hr before cell harvest. The cells were harvested onto glass fiber filter paper using an automatic cell harvester. The filters were counted as counts per minute (cpm) in a liquid scintillation cocktail of 3.62 g PPO and 0.08 g Bis-MSB (Omuni-fluor, New England Nuclear, Boston, Mass., USA) in one liter toluene (Dotsai scintisol, Wako Pure Chemical Industries Ltd., Tokyo, Japan) using a liquid scintillation spectrometer (Beckman LS-4800. Beckman Instruments Inc., Irvine, Ca, USA).

Statistical analysis: Statistical evaluation of differences between groups was done by the Student’s t-test.

Results

Effect of food restriction on longevity: GF and SPF mice were either full-fed or given food restricted to 80% of full-fed intake to the end of their normal life span. Survival data for all groups are presented in Table 1. At 110 weeks of age, full-fed and food-restricted GF mice had survival values of 9.5% and 36.4%, respectively; corresponding values for SPF mice were 4.1% and 53.6%. The survival values

| Table 1. Survival rates of GF and SPF mice either full fed or given restricted diets |
|-----------------|-----------------|-----------------|
| Microbial status | Dietary group   | Percent survival |
|                  |                 | after 55 week   | 110 week |
| GF               | Full-fed (21) a | 90.5 b          | 9.5 c    |
|                  | Restricted (22) | 100.0           | 36.4     |
| SPF              | Full-fed (49) a | 63.3 b          | 4.1 c    |
|                  | Restricted (28) | 100.0           | 53.6     |

a: Numbers in parentheses indicate number of animals
b: p<0.05 Full-fed vs food-restricted
c: p<0.01 Full-fed vs food-restricted
Fig. 1. Effect of food restriction on Con A (5 µg/ml) mitogenesis of spleen cells from GF (A) or SPF (B) mice. The number of mice in each group were three to six except for the 90-week-old GF-F and 105-week-old GF-R groups which were two. The results of mitogenic response were shown as the stimulation index (SI) which was calculated by the following formula and indicated as mean ± S.E. [6-3H] thymidine uptake in unstimulated lymphocytes: 1,628±56 (GF-F), 1,709±136 (GF-R), 1,780±130 (SPF-F), and 1,807±130 (SPF-R) in 25-week-old; 1,769±213 (GF-F), 1,769±213 (GF-R), 1,780±130 (SPF-F), and 1,807±130 (SPF-R) in 40-week-old; 1,653±302 (GF-F), 1,479±286 (GF-R), 1,745±216 (SPF-F), and 1,597±95 (SPF-R) in 70-week-old; 979 (GF-F), 1,736±165 (SPF-F), and 1,736±165 (SPF-R) in 90-week-old; 1,324 (GF-R) in 105-week-old; 1,298±101 (SPF-R) in 140-week-old.

\[ \text{SI} = \frac{\text{cpm of cells stimulated with Con A}}{\text{cpm of unstimulated cells}} \]

Fig. 2. Effect of food restriction on LPS (5 µg/ml) mitogenesis of spleen cells from GF (A) or SPF (B) mice. The number of mice in each group were three to six except for the 90-week-old GF-F and 105-week-old GF-R groups which were two. The results of mitogenic response were shown as the stimulation index (SI) which was calculated by the following formula and indicated as mean ± S.E. [6-3H] thymidine uptake in unstimulated lymphocytes: 1,628±56 (GF-F), 1,709±136 (GF-R), 1,780±130 (SPF-F), and 1,807±130 (SPF-R) in 25-week-old; 1,769±213 (GF-F), 1,769±213 (GF-R), 1,780±130 (SPF-F), and 1,807±130 (SPF-R) in 40-week-old; 1,653±302 (GF-F), 1,479±286 (GF-R), 1,745±216 (SPF-F), and 1,597±95 (SPF-R) in 70-week-old; 979 (GF-F), 1,736±165 (SPF-F), and 1,736±165 (SPF-R) in 90-week-old; 1,324 (GF-R) in 105-week-old; 1,298±101 (SPF-R) in 140-week-old.

\[ \text{SI} = \frac{\text{cpm of cells stimulated with LPS}}{\text{cpm of unstimulated cells}} \]
Fig. 3. Effect of food restriction on in vitro PFC response of spleen cells from GF (A) or SPF (B) mice to SRBC. The number of mice in each group were three to six except for the 90-week-old GF-F and 105-week-old GF-R groups which were two. S. E. are indicated by bars.

Discussion

In the present study, we have shown some effects of GF status and mild food restriction on the aging process. In this experiment, GF-F mice survived longer than SPF-F mice. This result was in agreement with that of other investigators [3, 15]. The longer life span of the GF-F mice may be explained in part by the absence of infectious disease. In both GF and SPF mice, food-restricted mice (to 80% of full-fed controls) survived longer than full-fed mice. This result was in agreement with Snyder et al. [19, 20] reporting longer survival for food-restricted GF and CV rats than their full-fed counterparts. However, contrary to expectations, the life span of the GF-R mice was shorter than that of the SPF-R mice in our study. Our data indicate that survival was enhanced to a greater extent by food restriction alone than by the combination of GF status with food restriction.

In the study presented here, in both GF and SPF mice, mitogenic responses to Con A and LPS reached a peak later in life in food-restricted mice. They showed lower PFC responses early in life and generally higher later on as compared with the full-fed rodents. These findings agree with the conclusions originally drawn by Walford et al. [23] that food restriction can delay age-proportional decline in immune function and extend life span. Our data indicates that diet restriction may be delaying maturation of the immune system, extending youthful immunological function in both GF and SPF mice.

The immune system plays an integral role for food-restricted mice were significantly higher than those of full-fed mice (p < 0.01).

Mitogen responses: The effects of food restriction on the responses of spleen cells to Con A and LPS in both GF and SPF mice are shown in Figs. 1 and 2. At 25 weeks of age, the SPF-F mice showed more vigorous proliferative responses to Con A and LPS than the SPF-R mice (p < 0.01). This difference was also seen in GF mice in response to LPS (p < 0.01), but not to Con A. In both GF and SPF mice, mitogen-induced T-cell and B-cell proliferation decline sharply with age in full-fed mice, whereas the splenocytes of food-restricted GF and SPF mice exhibited the highest rates of proliferation at 40 weeks of age.

In vitro PFC responses: Figure 3 shows data comparing the number of PFC generated in spleen cell cultures from full-fed and food-restricted GF and SPF mice after in vitro spleen cell stimulation with SRBC. Spleen cells from 25-weeks-old full-fed mice developed higher numbers of PFC by in vitro stimulation than those obtained from the food-restricted mice. However, starting at 40 weeks of age, a marked decline was seen in this response, whereas at 40 weeks of age, spleen cells from GF-R and SPF-R mice showed a high response to SRBC in the in vitro assay before declining in further weeks.
in the defense of the body against microorganisms and neoplasms. Advancing age produces declining immunoresponsiveness. There is an increased incidence of malignancy and age-related disease that occurs with immunological senescence [6,7,11,24]. It has been demonstrated that reduction of the food intake in rats extended life span with concomitant reduction of spontaneous tumors [22,27,29]. Pollard et al. [14] reported that food restriction reduced the incidence of methylazoxymethanol–induced intestinal tumors. Another investigator, Weindruch et al. [27] has suggested that the immunological consequences of food restriction may contribute to effects on longevity and late-life spontaneous cancer. Our data indicated tendencies of higher immunological function in old age among the food-restricted mice who also subsequently outlived their full-fed counterparts. This supports the explanation that stronger immunological functions in such old mice probably work to inhibit incidence of tumors and other age-related diseases.

The mitogenic and PFC responses of GF mice were seen to both peak and remain lower than those of SPF mice. This data is in agreement with Webb et al. [25] and Ohwaki et al. [13]. The reason for this lower responsiveness to immune function in GF mice is not clear, but it may concern with the absence of bacterial flora in GF mice. GF–F mice outlived SPF–F mice, but GF–R mice had shorter life span than the SPF–R mice. We are thus faced with the question, why the combination of GF status with food restriction did not prolong the life span of mice compared to food restriction alone. The reason for this discrepancy is not clear, but one possible consideration is that the lower immunological activity in old GF–R mice is probably not sufficient for inhibiting incidence of spontaneous tumors. This notion seems in agreement with the report by Pollard and coworkers [16] who showed spontaneous tumor development in aging CV–F L–W and GF–F L–W rats, but less incidence in food-restricted CV–R L–W rats.

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References


無菌およびSPFマウスの免疫機能に及ぼす
制限食の影響

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無菌およびSPFマウスの老化過程における免疫機能に及ぼす制限食の影響について検討した。実験動物は雄のICR系無菌およびSPFマウスを用い、各自自由摂取群と制限食群とに分けた。制限食の開始時期は生後5週とし、制限食群の食餌は自由摂取群の摂取量の80%（4.5g/日）を每日与えた。平均寿命が最も延長した群はSPFマウス制限食群、次いで無菌マウス制限食群、無菌マウス自由摂取群、SPF自由摂取群であった。この結果、無菌状態と制限食の組合せでは顕著な寿命延命効果は認められなかった。一方、各群の加齢に伴う脾臓リンパ球のマイトゲン（Con A, LPS）に対する免疫応答能は、無菌およびSPFマウスともに制限食群は自由摂取群に比べて高い反応性を示した。同様に、免疫応答の結果、抗原誘発機能も同様の傾向がみられた。これらの成績から、食餌制限は加齢に伴う免疫機能の低下を抑制している可能性が示唆された。