CHAPTER 23

Aging, Dietary Restriction, and Cancer in a Germ-Free Animal Model

D.L. Snyder[1,2], B.S. Wostmann[1], and M. Pollard[1]

Introduction

Freedom from infectious disease and from tumors induced by microbial carcinogens or by viruses has made germ-free (GF) animals a valuable tool in cancer and aging research. Gordon et al. (1966) suggested that protection from the deleterious effects of microbes on the aging host would permit a better view of the endogenous aging process. His research showed that GF mice lived at least 8 months longer than conventional (CV) mice (24 vs. 16). Research by Morris Pollard at the University of Notre Dame's Lobund Laboratory showed that GF rats outlived CV rats (Pollard 1971) and spontaneously developed liver tumors (Pollard and Luckert 1979) and prostate adenocarcinomas (Pollard 1973). Young GF rats and mice have lower resting oxygen consumption, cardiac output, and reduced heart size (Wostmann 1975), and adult GF rats have reduced body size (Snyder and Wostmann 1987) when compared to CV rats. The longer life span and reduced body size of GF rats are traits similar to those reported for long-lived diet-restricted (DR) rats. Pollard and Wostmann (1985) examined the effect of DR on cancer development and aging in GF Lobund-Wistar (L-W) rats. Ten GF L-W rats restricted to approximately 70% of daily food intake lived well beyond any ad libitum-fed CV or GF L-W rats and showed few of the tumors common to old L-W rats.

The results of Pollard and Wostmann's early study initiated the much larger Lobund Aging Study at the University of Notre Dame. Designed to determine what pathologies would develop over the life span of ad libitum and DR CV and GF L-W rats, the study included investigators outside of the university who had interests in aging and its associated pathologies and biochemical changes. The present article is intended only to highlight the findings of the Lobund Aging Study. An earlier account of this study was published in the proceedings of the

[1]Lobund Laboratory, University of Notre Dame, Notre Dame, IN 46556, USA
[2]Current address: Center for Gerontological Research, The Medical College of Pennsylvania, 3300 Henry Avenue, Philadelphia, PA 19129, USA

L. Fishbein (ed.), *Biological Effects of Dietary Restriction*

symposium on the effects of dietary restriction on aging and disease in germ-free and conventional Lobund-Wistar rats (Snyder 1989).

Methods

Germ-free L-W rats were first propagated at the University of Notre Dame in 1958. The CV breeding colony was derived from the GF colony, and at regular intervals GF males and females are added to the CV colony to maintain close genetic proximity. Only male rats were used in this study. The GF rats were housed in plastic or steel isolators and were maintained using routine gnotobiotic procedures (Subcommittee on Standards for Gnotobiotics 1970). The CV rats were housed in plastic isolators to limit exposure to the local environment. CV isolators were opened only to introduce feed and water and to clean the cages. Ad libitum-fed rats (GF-F and CV-F) were housed four to a cage in commercial plastic boxes. DR rats (GF-R and CV-R) were taken from their respective breeding colonies at 6 weeks of age and housed individually in plastic cages. All rats were fed steam-sterilized natural ingredient diet L-485, our colony diet since 1968 (Kellogg and Wostmann 1969). The DR rats were never allowed more than 12 g of diet per day. This method of feeding becomes restrictive at about 8 weeks of age and results in a 30% reduction in feed intake in adult rats (Snyder and Wostmann 1987). A complete description of the housing and diet is provided in Snyder et al. (1990).

Rats were killed if they were in a moribund condition due to a palpable tumor or had severe weight loss over several months. Healthy rats were selected for killing from 3 to 30 months of age for measurement of physiological parameters related to aging. After an overnight fast, each rat was anesthetized with halothane and blood was removed from the exposed heart with a needle and syringe. Blood was allowed to clot for 30 min and then centrifuged. The serum was frozen at $-70°C$ in individual aliquots for later analysis. Individual tissues were quickly removed, weighed, and frozen in liquid nitrogen or preserved according to the protocol of each investigator receiving tissue samples. Rats were examined for tumors and tissue samples were fixed for histological examination.

Results and Discussion

Each of the four experimental groups had a characteristically different pattern of growth. Up to 4 months of age the GF-F rats grew at the same rate as the CV-F rats, but after 5 months the GF-F rats had a slightly slower rate. By 18 months the average weight of CV-F rats was just over 480 grams, but the GF-F rats averaged just over 450 grams. Since the cecum of GF rats is 20 grams larger than that of CV rats, the corrected difference in body weights between GF-F and CV-F rats may be considered closer to 50 grams. The CV-R and GF-R rats grew at a similar rate, but adult GF-R rats tended to be slightly heavier. Further details on the growth of GF and CV L-W rats are available in Snyder and Wostmann (1987).

One hundred CV-F, 88 CV-R, 96 GF-F, and 127 GF-R rats were used to determine the survival characteristics of the male L-W rat. The length of life of the four experimental groups closely followed their adult body weights. The median length of life of the heavier CV-F and GF-F rats were 31.0 and 33.6 months, respectively, while the lighter CV-R and GF-R rats were 38.6 and 37.8 months, respectively. The 10th percentile and maximum survival ages were CV-F: 36.3/38.9, GF-F: 39.0/40.5, CV-R: 43.9/50.9, and GF-R: 44.8/47.4. Statistical analysis showed that the survival of each of the DR groups was different from that of the corresponding ad libitum-fed groups, that the survival of the GF-F rats was different from that of the CV-F rats, and that the survival of the GF-R and CV-R rats were equivalent (Snyder et al. 1990). The small increase in life span between GF-F and CV-F rats may be due partly to the lack of prostatitis in GF rats and a natural DR state as indicated by a smaller adult body size. GF status was without additional benefit when food intake and final body weights were similar.

Most tumors do not appear in L-W rats until after 30 months of age. The majority of these tumors develop spontaneously in the liver, the adrenal medulla, and the mammary glands. Although the frequencies of these pathologies were lower in the CV-R, GF-F, and GF-R rats than in the CV-F rats, the major effect of DR and GF life was to delay the age of occurrence of these tumors. Hepatomas were found in 58% of moribund CV-F rats at a mean age of occurrence of 32.5 months. The corresponding figures for hepatomas in the other three experimental groups were CV-R: 34% and 41.1, GF-F: 47% and 37.3, and GF-R: 42% and 38.4. Prostate adenocarcinomas were found in 22% of moribund CV-F rats at a mean age of occurrence of 28.1 months. The corresponding figures for prostate adenocarcinomas in the other three experimental groups were CV-R: 7% and 40.0, GF-F: 7% and 22.0, and GF-R: 6% and 37.0. DR also reduced the incidence of prostatitis in CV rats and delayed the mean age of occurrence from 23.3 to 41.0 months.

It should be noted that nephrosis was not a common finding in any of the healthy or moribund L-W rats examined in this study. When it occurred, it was usually considered mild and was never the cause of death. The use of soy protein rather than casein protein along with a natural resistance to kidney disease seems to have prevented nephrosis in L-W rats. A complete description of the pathological finding of the Lobund Aging Study has been reported in Pollard et al. (1989) and Snyder et al. (1990).

Healthy and moribund L-W rats were examined for cardiomyopathy and pituitary adenomas by outside investigators. Cornwell and Thomas (1989) reported that the occurrence of cardiac fibrosis was age related and most extensive after 30 months of age. DR significantly reduced the amount of fibrosis at all ages. Sano et al. (1989) reported that gonadotroph nodules were common in ad libitum-fed L-W rats older than 26 months, but the development of this age-associated lesion was delayed by DR and GF status parallel to prolongation of life span.

Thus a restriction of only 30% in food intake caused a considerable delay in the development of a broad array of pathologies common to L-W rats and extended the life span by approximately 8 months in CV rats and 4 months in GF rats.

A number of age-associated physiological changes were seen in the rats examined during the Lobund Aging Study. Serum levels of thyroid stimulating hor-

mone (TSH), thyroxine (T4), triiodothyronine (T3), prolactin (PRL), and testosterone (T) have been reported for the 4 experimental groups from 6 to 30 months of age (Snyder et al. 1988). TSH and T4 levels were slightly higher in the GF-F and GF-R rats, both hormones declined gradually from the youngest to the oldest rats examined, and DR had no effect on either hormone. T3 levels were also unaffected by DR and did not change over time in CV rats. Within the GF animals, DR had no effect, young rats had considerably higher T3 levels, and old rats had considerably lower T3 levels than CV rats of the same age. PRL levels were generally similar between CV and GF rats, but DR prevented the age-associated rise in PRL levels seen in ad libitum-fed rats. T levels showed a gradual decline from adult to old age in all rats, but DR dramatically raised T levels at all ages. There was no reduction in testes size in DR rats when compared to the ad libitum-fed rats. These results indicated that a reduction in thyroid hormones and in reproductive function was not necessary to promote an extended life span in DR rats.

Serum insulin, blood glucose, serum cholesterol, and serum triglycerides in 6–30 month old rats were reported by Snyder and Towne (1989). Ad libitum-fed rats had slightly higher serum insulin levels, but there were no differences due to age or GF status. Serum glucose levels were similar in all rats except for a tendency to high levels in the oldest CV-F and GF-F rats. Serum cholesterol and triglyceride levels were higher in CV rats, reduced by DR and increased with age. A recently completed study at Lobund Laboratory using only CV L-W rats has shown that DR will prevent the age-associated increase in glycosylated hemoglobin levels. In this study serum glucose levels were similar in CV-F and CV-R rats from 6 to 30 months, but while CV-F rats showed a steady increase in glycosylated hemoglobin after 18 months of age, there was no increase with age in CV-R rats.

Although DR had very little effect on growth-regulating hormones such as insulin, T4, and T3, DR did significantly reduce serum levels of somatomedin-D/insulin-like growth factor-I (SMD-C) in the L-W rats in this study (Prewitt and D'Ercole 1989). SMD-C is a growth-hormone-dependent anabolic peptide that has a high correlation with nitrogen metabolism and body weight. Serum SMD-C levels were highest in the CV-F rats from 5 to 18 months of age but fell to levels equal to the DR rats at 30 months of age. GF status also reduced serum SMD-C levels but not to the extent seen in DR rats.

Age-associated changes in the bone were observed by two investigators. Nishimoto et al. (1990) found declines in serum bone γ-carboxyglutamic acid (Gla) protein, and matrix concentrations of calcium, magnesium, and bone Gla protein in all four experimental groups as they aged. The decline in these parameters, which indicates a loss of bone mineral and a reduction in bone mineralization with age, was not altered by DR or GF status. Rosen et al. (1989) showed a significant loss of alveolar bone with age in all four experimental groups that was not altered by DR or GF status. These results are contradictory to those of Kalu et al. (1988) who reported that DR appeared to stop an age-associated decrease in bone density in Fischer 344 rats. It is possible that nephrosis caused by a casein diet and then corrected through DR contributed to the effects seen in the Fischer

344 rats. DR may have very little direct effect on age-associated changes in bone metabolism when nephrosis is absent as in the L-W rats.

Several investigators examined the hypothesis that DR alters the accumulation of oxidative and free radical-initiated damage that may be responsible for the degenerative changes associated with aging. Chen and Lowry (1989) found that the liver activity of three enzymes which are part of the antioxidative defense system was enhanced by DR in the CV L-W rats. Superoxide dismutase (responsible for removing superoxide radicals), catalase, and selenium-dependent glutathione peroxidase (both decompose cellular hydrogen peroxide) were enhanced in 30-month-old CV-R rats when compared to 30-month-old CV-F rats. GF status, however, tended to lower the activity of superoxide dismutase and catalase when compared to CV rats. Selenium-dependent glutathione peroxidase activity, however, was enhanced in both GF-F and GF-R rats. The lower metabolic rate of GF rats may have reduced the production of free radicals and therefore lowered the activity of the enzymes responsible for removing free radicals. Lang et al. (1989) measured whole blood levels of glutathione in 6- to 40-month-old rats. Glutathione serves as the substrate in the enzymatic reaction that decomposes hydrogen peroxide. DR increased blood glutathione levels by 20%–100% at all ages. After a peak at 18 months blood glutathione declined with age, but the decline was slower in DR rats. Starke-Reed (1989) looked for the accumulation of oxidized proteins in the testes of aging L-W rats. There was a large increase in oxidized protein between 6 and 18 months of age in the CV-F and CV-R rats, but at 30 months the amount remained high in the CV-F rats and declined in the CV-R rats. The levels of oxidized proteins were lower in both groups of GF rats when compared to CV rats. Because of its susceptibility to oxidation, glutamine synthetase activity was also measured in the testes. The CV-F rats showed a decline in activity of 40% between 6 and 30 months, but there was no change in the other three groups. This study also suggests that GF status imparts a protection against free radical damage that is similar to that provided by DR. From the three studies mentioned above it would appear that DR does enhance the cellular mechanisms which destroy free radicals once they are produced. Within the GF-F rats such protection may also be present, but it is not additive with DR.

Other investigators in the Lobund Aging Study have examined the influence of DR on immune function, body composition, gastrointestinal function, muscle enzymes, and microsomal monooxygenases. The complete reports of these and other studies will soon be published. Hopefully the integration of all the findings from the Lobund Aging Study will help in the search for a universal hypothesis for the action of DR on the aging process.

Conclusion

Our study confirms the ability of DR to extend life span in the rat by showing that rats which are virtually free of kidney disease (due to consumption of a casein-free diet) and free of infectious disease (from living in a GF environment) still

receive considerable benefit from reduced dietary intake. It is also apparent from the Lobund Aging Study that GF status, even with its reduced metabolic rate, has a relatively small effect on life span when compared to DR.

The GF animal has been suggested as an ideal model for aging research because of the freedom from microbial interference during aging, but the cost of maintaining GF animals for extended periods is prohibitive. Our study has shown that conventional L-W rats derived from a clean breeding colony, housed in minimal barrier isolation, and fed a natural ingredient diet will live almost as long as GF L-W rats. Freedom from kidney disease and the presence of many age-associated pathologies rather than one overriding cause of death may make the L-W rat an ideal animal for aging research.

References

Chen LH, Lowry SR (1989) Cellular antioxidant defense system. In: Snyder DL (ed) Dietary restriction and aging. Liss, New York, pp 247–256

Cornwell GG, Thomas BP (1989) Cardiac fibrosis in the aged germfree and conventional Lobund-Wistar rat. In: Snyder DL (ed) Dietary restriction and aging. Liss, New York, pp 69–74

Gordon HA, Bruckner-Kardoss E, Wostmann BS (1966) Aging in germ-free mice: life tables and lesions observed at natural death. J Gerontol 21:380–387

Kalu DN, Masoro EJ, Yu BP, Hardin RR, Hollis BW (1988) Modulation of the age-related hyperparathyroidism and senile bone loss in Fischer rats by soy protein and food restriction. Endocrinology 122:1847–1854

Kellogg TF, Wostmann BS (1969) Stock diet for colony production of germfree rats and mice. Lab Anim Care 19:812–814

Lang CA, Wu W, Chen T, Mills BJ (1989) Blood glutathione: a biochemical index of life span enhancement in the diet restricted Lobund-Wistar rat. In: Snyder DL (ed) Dietary restriction and aging. Liss, New York, pp 241–246

Nishimoto SK, Padilla SM, Snyder DL (1990) The effect of food restriction and germfree environment on age-related changes in bone matrix. J Gerontol Biol Sci 45:B164–168

Pollard M (1971) Senescence in germfree rats. Gerontologia 17:333–338

Pollard M (1973) Spontaneous prostate adenocarcinomas in aged germfree Wistar rats. J Natl Cancer Inst 51:1235–1241

Pollard M, Luckert PH (1979) Spontaneous liver tumors in aged germfree Wistar rats. Lab Anim Sci 29:74–77

Pollard M, Wostmann BS (1985) Aging in germfree rats: the relationship to the environment, diseases of endogenous origin, and to dietary modification. In: Archibald J, Ditchfield J, Rowsell HC (eds) The contribution of laboratory animal science to the welfare of man and animals. Fischer, New York, pp 181–186

Pollard M, Luckert P, Snyder DL (1989) Prevention of prostate cancer and liver tumors in L-W rats by moderate dietary restriction. Cancer 64:686–690

Prewitt TE, D'Ercole AJ (1989) Modest dietary restriction and serum somatomedin-C/insulin like growth factor-I in young, mature and old rats. In: Snyder DL (ed) Dietary restriction and aging. Liss, New York, pp 157–162

Rosen S, Strayer M, Glocker W, Marquard J, Beck FM (1989) Effects of aging, diet restriction and microflora on oral health in humans and animals. In: Snyder DL (ed) Dietary restriction and aging. Liss, New York, pp 75–85

Sano T, Kovacs K, Stefaneanu L, Asa S, Snyder DL (1989) Spontaneous pituitary gonadotroph nodules in aging male Lobund-Wistar rats. Lab Invest 61:343–349

Snyder DL (ed) (1989) Dietary restriction and aging. Liss, New York

Snyder DL, Towne B (1989) The effect of dietary restriction on serum hormone and blood chemistry changes in aging Lobund-Wistar rats. In: Snyder DL (ed) Dietary restriction and aging. Liss, New York, pp 135–146

Snyder DL, Wostmann BS (1987) Growth rate of male germfree Wistar rats fed ad libitum or restricted natural ingredient diet. Lab Anim Sci 37:320–325

Snyder DL, Wostmann BS, Pollard M (1988) Serum hormones in diet-restricted gnotobiotic and conventional Lobund-Wistar rats. J Gerontol Biol Sci 43:B168–173

Snyder DL, Pollard M, Wostmann BS, Luckert P (1990) Life span, morphology, and pathology of diet-restricted germ-free and conventional Lobund-Wistar rats. J Gerontol Biol Sci 45:B52–58

Starke-Reed PE (1989) The role of oxidative modification in cellular protein turnover and aging. In: Snyder DL (ed) Dietary restriction and aging. Liss, New York, pp 269–276

Subcommittee on Standards for Gnotobiotics, Committee on Standards, Institute of Laboratory Animal Resources, National Resource Council (1970) Gnotobiotes, standards and guide lines for breeding, care and management of laboratory animals. National Academy of Sciences, Washington

Wostmann BS (1975) Nutrition and metabolism of the germfree mammal. World Rev Nutr Diet 22:40–92