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Effects of food restriction on aging: Separation of food intake and adiposity

(longevity/collagen/biomarkers/obesity/immune response)

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ABSTRACT **Restricted feeding of rodents increases longevity, but its mechanism of action is not understood. We studied the effects of life-long food restriction in genetically obese and normal mice of the same inbred strain in order to distinguish whether the reduction in food intake or the reduction in adiposity (percentage of fatty tissue) was the critical component in retarding the aging process. This was possible because food-restricted obese (*ob/ob*) mice maintained a high degree of adiposity. In addition to determining longevities, changes with age were measured in collagen, immune responses, and renal function. Genetically obese female mice highly congenic with the C57BL/6J inbred strain had substantially reduced longevities and increased rates of aging in tail tendon collagen and thymus-dependent immune responses, but not in urine-concentrating abilities. When their weight was held in a normal range by feeding restricted amounts, longevities were extended almost 50%, although these food-restricted *ob/ob* mice still had high levels of adiposity, with fat composing about half of their body weights. Their maximum longevities exceeded those of normal C57BL/6J mice and were similar to longevities of equally food-restricted normal mice that were much leaner. Food restricted *ob/ob* mice had greatly retarded rates of collagen aging, but the rapid losses with age in splenic immune responses were not mitigated. Thus, the extension of life-span by food restriction was inversely related to food consumption and corresponded to the aging rate of collagen. These results suggest that aging is a combination of independent processes; they show that reduced food consumption, not reduced adiposity, is the important component in extending longevity of genetically obese mice.**

By far the most effective method of increasing longevities in mammals is food restriction (1, 2). Substantial increases have been reported in life-spans of mice and rats whose food intake was restricted by a variety of methods (3–6), and this increase has been attributed to reduced adiposity. These studies have been thought to be relevant to human beings, because there was a continuous, direct relationship between obesity and mortality over a wide range of body weights reported in a very large study by The Society of Actuaries (7). Recently this relationship has been challenged. In surveying relevant longitudinal studies of human beings, Andres found that the lowest mortality rates were shown not by lean people but by individuals 20–30% heavier than their conventionally defined "desirable weights" (8). These conflicting findings may result from the fact that food intake is restricted in the rodent experiments, while the data on human beings, derived from height-to-weight ratios, is a rough estimate of adiposity. Two studies have suggested that adiposity is not correlated with longevity in rats (9, 10); however, the undefined genotypes in one study (9) and the small number examined for this correlation in the other (10) prevented their conclusions from being definitive.

We studied the relationship between food intake, adiposity, and aging by using food-restricted genetically obese *ob/ob* mice. These animals gain weight very rapidly and become extremely obese (11–15). Their life-spans are reduced, but an early report indicated that they could be extended by food restriction (14). Mice of the *ob/ob* genotype maintain high percentages of body fat even when their food intake is restricted, because their metabolism is intrinsically different from that of normal mice (11–15). Thus, they may be used to study the effects of food intake on the aging process separately from the effects of adiposity.

In the present studies, genetically obese and normal mice of the C57BL/6J (B6) inbred strain were fed according to two different patterns: unlimited access to food, or restriction to a constant amount. There were dramatic differences in food consumption and in patterns of weight gain and degrees of adiposity in the four types of mice. We studied their longevities, the rate of change with age in tail tendon collagen denaturation rates, urine-concentrating abilities, and thymus-dependent immune responses.

MATERIALS AND METHODS

Mice. The B6 +/+ and B6 *ob/ob* female mice used in the study were produced and raised at The Jackson Laboratory, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Genetically obese (*ob/ob*) and normal mice were distinguished at weaning by differences in weight. Mice of each genotype were divided into two groups: fed mice were allowed unlimited access to food (fed ad lib), while restricted mice were given their rations each day in a single feeding between 12:00 and 2:00 p.m., 5 days a week, with a double feeding on the sixth day and none on the seventh. These feeding regimens were continued from weaning, at 4 weeks of age, until death. The amount of food given to both types of restricted mice was the amount that maintained the weight of the restricted obese mice at the level of the fed normal mice. This was about two-thirds of the amount of food consumed by these normal mice, and less than half of that consumed by fed obese mice (Table 1). Four mice were caged in each side of a double-sided box, and each restricted mouse was given at least one portion of a food pellet when fed. Percentages of body water were determined by weight loss after lyophilization of homogenized aliquots, and percentages of fat were measured from the weight loss of the dehydrated aliquots on extraction with 1-butanol/diisopropyl ether (40:60, vol/vol) as described (16).

Mice in this study were housed in an isolated, environmentally controlled room under positive pressure with filtered air, room temperatures at 22 ± 2°C, and lighting from 6:00 a.m. to 6:00 p.m. The mice were all fed the same pas-

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Abbreviations: B6, C57BL/6J; *ob/ob*, obese mice that are homozygous for the obesity gene; SRBC, sheep erythrocytes; PHA, phytohemagglutinin.

teurized diet (96WA, Emory Morse; Guilford, CT), containing 357 kcal/100 g, 22% protein, 7% fat, and 50% nitrogen-free extract (NFE, which is mostly carbohydrates). Further details about animal husbandry and diets are given in ref. 17. Mice were not exposed to pathogens known to affect longevity, and a detailed description of the animal health monitoring for this colony has been published (18).

Measurement of Physiological Changes with Age. Aging rates of tail tendon collagen were measured by denaturation times of tendon fibers in 7 M urea at 45.0°C. A detailed description of this technique has been published (19). Urine-concentrating ability was measured by removing drinking water but not food and injecting mice intraperitoneally with 1.0 ml of 0.9% saline at approximately 12:00 noon. Urine was sampled 48 hr later by placing mice on a strip of clean Parafilm and gently palpating their abdomens, if necessary, to produce the urine sample. The urine osmolality was measured on a Wescor 5100 B vapor pressure osmometer, using 5-μl samples that had been diluted as necessary for accurate measurements.

Thymus-dependent immune responses were measured by using techniques similar to those reported (20, 21). Proliferative responses to the mitogen phytohemagglutinin (PHA) *in vitro* were measured in microplates, with 0.30 μg of PHA (Wellcome Reagents HA-17) and 5×10^5 spleen cells in a total volume of 0.20 ml of RPMI 1640 medium per well. After 62 hr, tritiated thymidine was added to each well. Plates were frozen 8 hr later and thawed, samples were precipitated and collected on glass fiber filters with a microplate harvester, and amounts of tritiated thymidine incorporation were measured by scintillation counting. Formation of plaques in a response to sheep erythrocytes (SRBC) *in vivo* was measured by a modified Jerne plaque assay. Numbers of direct anti-SRBC plaques were counted 5 days after the tested mice were immunized with 1×10^9 SRBC injected intravenously. In each experiment, young controls (3-month-old B6 +/+ mice) were tested, and the data on immune responses were adjusted to the percentage of the young control response. In all cases, reponses were calculated per spleen by multiplying the response of an aliquot of spleen cells by the number of such aliquots in the spleen.

RESULTS

Growth Rates, Body Composition, and Food Intake. When fed ad lib, *ob/ob* mice gained weight rapidly and became extremely heavy (Fig. 1*A*). Weight gain patterns for normal mice allowed unrestricted access to food (fed +/+) and for the restricted *ob/ob* mice were similar, except that the latter gained weight slightly faster during the first few weeks. Restricted +/+ mice were given the same amount of food as restricted *ob/ob* mice and weighed much less (Fig. 1*A*).

Body compositions and food intakes differed dramatically among the four groups (Table 1). Fed *ob/ob* mice ate the most, averaging 4.2 g per day, and about two-thirds of their body weight was fat. Restricted *ob/ob* mice, given 2.0 g of food per day, were much lighter but still maintained about one-half of their body weight as fat. Fed +/+ mice ate 3.0 g of food per day, and only about 20% of their body weight was fat, while restriced +/+ mice given the same amount of food as restricted *ob/ob* mice had only one-quarter as much fat (Table 1). Although the fed *ob/ob* mice used in studies of body composition happened to be lighter than those in Fig. 1*A*, they still averaged 40 g of fat, while restricted *ob/ob* mice averaged 13 g. This compares to 7 and 3 g for fed and restricted +/+ mice, respectively. Similar calculations show that nonfat body weights were lower for *ob/ob* than +/+ mice and were reduced by food restriction in both groups.

Aging of Collagen and a Renal Function. The rate of collagen aging was greatly accelerated in fed *ob/ob* mice and greatly retarded by food restriction (Fig. 1*B*). Collagen aged at the same rate in food-restricted *ob/ob* and +/+ mice, although the former had a much higher percentage of body fat. For example, denaturation times were similar for tendon fibers removed from the following groups: 265-day-old fed *ob/ob* mice, 425-day-old fed +/+ mice, and 550-day-old restricted *ob/ob* and restricted +/+ mice (Fig. 1*B*). Thus collagen aging rates decreased with the amount of food consumed; they were not affected by the degree of adiposity.

Changes with age in urine-concentrating abilities were erratic. They tended to decline most rapidly in fed +/+ mice

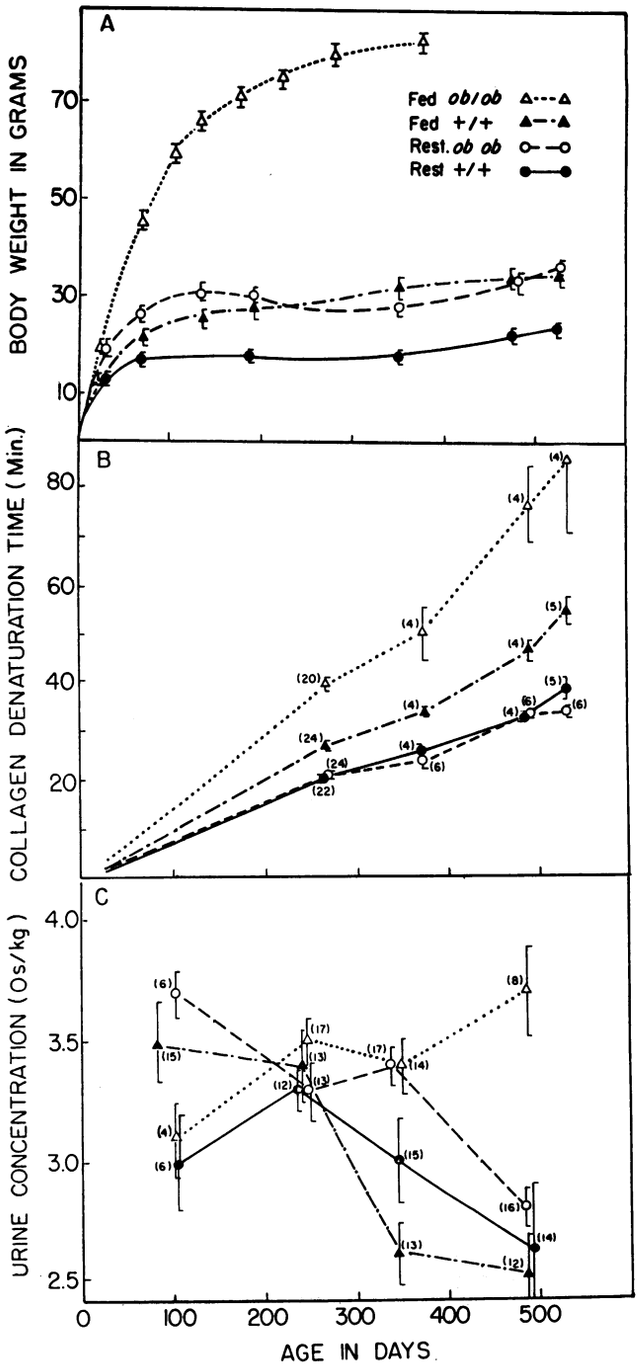

FIG. 1. Changes with age in body weights (*A*), tail tendon collagen denaturation times (*B*), and urine-concentrating abilities (*C*) are given for female B6 genetically obese (*ob/ob*) and normal (+/+) mice, allowed unlimited access to food (Fed) or allowed to eat only restricted amounts 6 days a week (Rest.). Means are plotted, and bars show ±SEM, with numbers of mice tested at each point shown in the figure, except that 4–6 mice were tested at each point in *A*. Os, osmoles.

Table 1. Adiposity and food intake of fed and restricted genetically obese and normal mice

Group	Food, g/day	Body wt, g	Fat, % wt
Fed *ob/ob*	4.2	59 ± 5	67 ± 5
Fed +/+	3.0	*30 ± 1	*22 ± 6
Rest. *ob/ob*	2.0	*28 ± 2	48 ± 1
Rest. +/+	2.0	20 ± 2	*13 ± 3

All mice were B6 females. Fed groups had unlimited access to food, while restricted (Rest.) groups were given limited amounts once daily 6 days per week. Body weights and fat compositions are given for 6- to 12-month-olds, $n = 4$–8 per group. The tested individuals did not differ significantly over this age range. Food intake adjusted to mean g per day per mouse is given for 1 week at 4.5 months of age with 8 mice per group. Results are given as mean ± SEM; means not significantly different from each other are preceded by an * ($P < 0.05$, Student–Neuman–Keuls multiple-range test).

and more slowly in restricted *ob/ob* and restricted +/+ animals (Fig. 1*C*). Surprisingly, urine-concentrating abilities did not decline with age in fed *ob/ob* mice, whose urine concentrations after 2 days of dehydration were the highest in groups tested from 250 to 500 days of age.

Thymus-Dependent Immune Responses and Longevities. In *ob/ob* mice 550–580 days of age, responses to SRBC *in vivo* and to PHA *in vitro* were only about 10% of young normal levels (Table 2). Surprisingly, the food restriction treatment failed to improve the levels of these immune responses per spleen in *ob/ob* mice at 550–580 days of age. Immune responses in normal mice of that age had also declined significantly from young levels, but less than the decline shown by the *ob/ob* mice. Food restriction had slight beneficial effects on immune responses in 550- to 580-day-old +/+ mice (Table 2). Since these responses were calculated per spleen, one reason that restricted *ob/ob* mice had low responses is because they had very small spleens. Spleen weights in these mice at 550–580 days of age were as follows: fed *ob/ob*, 75 ± 17 mg; fed +/+, 150 ± 8 mg; restricted *ob/ob*, 36 ± 3 mg; and restricted +/+, 78 ± 4 mg. Spleen weights are given as mean ± SEM for groups of 10–14 individuals.

Food restriction increased the mean and maximum longevities of *ob/ob* mice 56% and 46%, respectively (Table 2). It had little effect on the mean longevity of +/+ mice but increased their maximum longevity 32%. This suggests that food restriction with the diet used reduced levels of one or more essential micronutrients to a point that increased mortality of some +/+ mice. The degree of restriction was greater in the case of *ob/ob* mice, since they were given about one-half of the 4.2 g per day consumed when fed ad lib, while +/+ mice were given about two-thirds of their ad lib consumption (Table 1).

DISCUSSION

Food Intake, Adiposity, and Collagen Aging. To separate the effects of food intake and adiposity, we studied food-restricted B6 *ob/ob* mice, weighing about the same as normal mice, but with almost 50% adipose tissue (Table 1). Since these mice had more than twice as high a percentage of fat as was observed in fed normal mice but were fed only two-thirds of the amount consumed by normals (Table 1), the effect of adiposity was clearly separated from that of food intake. Collagen aging was important to measure, because it is accelerated in human diabetes, as well as in an experimentally induced rat diabetes and in *ob/ob* mice (22–24), and its rate is known to be retarded by food restriction (25–27). In the experiments reported here, we confirmed that the rate of collagen aging is greatly accelerated by genetic obesity and retarded by food restriction. More importantly, we showed that collagen aged at the same rate in mice fed the same amount regardless of their degree of adiposity. Fig. 1*B* demonstrates that collagen aging is equally retarded in *ob/ob* and +/+ mice fed the same restricted amounts, although the +/+ mice had one-quarter the percentage of body fat that *ob/ob* mice had (Table 1). Thus, collagen aging is related to food intake rather than to adiposity.

Changes with Age in a Renal Function. The degree to which urine can be concentrated after dehydration is a simple measure for a major aspect of renal function. Maximum urine-concentrating ability has been shown to decline with age in normal rodents (18, 28–31). To our surprise, this function was not deleteriously affected by obesity; in fact, it did not decline in fed *ob/ob* mice, whose urine-concentrating ability was the highest in groups tested at 250 to 500 days of age (Fig. 1*C*). A possible explanation for this observation may be that the glomerular filtration rate in our obese mutant mice is enhanced similarly to the increase observed in B6 *db/db* mice (32). However, this was observed only in young B6 *db/db* animals; filtration rates dropped to normal values by 150 days of age. Futhermore, Meade *et al.* (33) showed that immune complexes were deposited in renal glomeruli of fed *ob/ob* mice much more rapidly than in normal mice. This specific evidence for renal damage, and the other physiological defects resulting from the gross obesity, make it interesting that fed *ob/ob* mice maintained high urine-concentrating abilities throughout their lives.

Immunological Aging. The decline with age in thymus-dependent immune responses has been extensively reviewed (34–39). We found that proliferative T-cell responses to the PHA mitogen *in vitro* and plaque-forming cell responses to SRBC *in vivo* were normal in young *ob/ob* mice, but they declined by 550 to 580 days of age to about 10% of normal responses, a much more severe decline than that shown by

Table 2. Effects of food restriction and genetic obesity on immune responses and longevity

Group	Immune response of old mice, % Y		Longevity, days			
	SRBC	PHA	Median	Mean	*n*	Maximum 10%
Fed *ob/ob*	7 ± 7	13 ± 7	552	526 ± 36	29	776–893
Fed +/+	22 ± 11	49 ± 12	799	771 ± 28	32	954–976
Rest. *ob/ob*	11 ± 7	8 ± 6	814	823 ± 46	39	1209–1307
Rest. +/+	59 ± 30	50 ± 11	850	810 ± 48	38	1089–1287

All mice were B6 females. Immune responses were measured at 550–580 days of age and are given as % Y (percentages of young control, which was 3-month-old fed +/+) responses per spleen, with $n = 8$–14 per group. SRBC (Jerne's plaque assay against SRBC *in vivo*) and PHA (mitogenic response to PHA *in vitro*) responses were tested. Young (3-month-old) fed *ob/ob* mice had responses similar to those of the young +/+ controls: 115 ± 19% and 79 ± 16% of young control values for SRBC and PHA, respectively. Actual responses of young +/+ controls were (27 ± 5) × 10^4 plaques and (254 ± 18) × 10^5 cpm per spleen, for SRBC and PHA, respectively. Results are given as means ± SEM, except that the range is given for longevities of the longest-lived 10%. All immune responses were significantly lower than young values, except the SRBC responses of restricted +/+ ($P > 0.05$, Student–Neuman–Keuls multiple-range test).

+/+ mice (Table 2). Decline in immune responses may be a basic aging process (34); they should at least be an indicator of individual health. In either case they should be correlated with longevity. Therefore, we were surprised when the food restriction treatment that was so beneficial to the longevity of *ob/ob* mice failed to improve their levels of immune responses at 550–580 days of age (Table 2). At this age, fed *ob/ob* mice were near the ends of their lives, so it is not surprising that their immune responses were low. However, immune responses in food-restricted *ob/ob* mice were equally low even though their life expectancies were much longer. Possibly, immune responses were not correlated to longevity because our animal colony is unusually free of pathogenic organisms (18). It is also possible that immune responses of cells were higher in organs other than spleen. The spleens of restricted *ob/ob* mice were unusually small, and responses were calculated per spleen.

Our data also suggest that the higher the levels of body fat, the more rapidly immune responses will decline with age. This suggestion is supported by a number of reports. Food restriction increased immune responses in aging normal mice (40–43); restricted mice in these studies almost certainly had reduced levels of body fat, as did ours (Table 1). Fernandes *et al.* (41, 42) have shown that autoimmunity develops more rapidly as dietary fat is increased. This is also consistent, since normal immune responses decline when autoimmune responses develop, and high levels of dietary fat would be expected to cause increased levels of body fat.

Finally, our data suggest that the aging rates of collagen, renal functions, and immune responses are not timed by a single process. These characteristics appear to age independently and are each affected differently by genetic obesity and food restriction. Aging thus may be a combination of many different processes, at least some of which may be affected independently by treatments designed to retard or reverse changes with age.

Food restriction in rodents retards changes with age in a number of physiological processes, in addition to the ones that we tested (44–48). These beneficial effects appear to contradict the findings in longitudinal studies of human beings that moderate rather than minimal levels of obesity are associated with minimal levels of mortality (7). However, our findings in this report suggest a way to resolve this contradiction. Individuals with a genetic obesity causing unusually efficient fat production had greatly extended longevities (Table 2) when food consumption was halved, although this still allowed more than twice normal percentages of body fat (Table 1). Longevities were related to food consumption rather than to the degree of adiposity. Recent studies have shown beneficial effects from food restriction with micronutrient supplementation in adult mice (49). Our work suggests that reducing food consumption may be beneficial even for individuals that retain high percentages of body fat.

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