Calorie Source, Calorie Restriction, Immunity and Aging of (NZB/NZW)F_1 Mice[1]

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ABSTRACT It is frequently stated that high fat diets are harmful with respect to nutrition and disease development. Herein, (NZB/NZW)F_1 autoimmune-prone mice were compared under the influence of different calorie intakes and different calorie sources. Decreased calorie intake prolonged life and delayed onset of glomerulonephritis. This influence of restriction of energy intake was greater than any influences of dietary energy source. Parameters affected strictly by restricted calorie intake were: *1*) longevity, *2*) delayed onset of glomerulonephritis, *3*) greatly decreased circulating immune complexes, *4*) decreased production of anti-DNA antibodies, *5*) increased thymocyte proliferation in response to exogenous Interleukin-2 (IL-2), *6*) increased IL-2 production by spleen cells stimulated by concanavalin A and *7*) marked increase of mixed lymphocyte reaction of spleen cells. Parameters affected both by restriction of calorie intake and by high sucrose content in full-fed mice and not obviously related to longevity and protection from glomerulonephritis were: *1*) plaque-forming cell response to sheep red blood cells in vitro and *2*) cytotoxic cell-mediated immune response generated by in vitro exposure to allogenic antigen. J. Nutr. *114*: 1884–1899, 1984.

INDEXING KEY WORDS calorie source • calorie restriction • energy intake • longevity • aging • glomerulonephritis • lupus • autoimmunity • (NZB/NZW)F_1 mice • immune function

The earlier reports by McCay et al. (1–3) and of Ross and Bras (4), on the effect of dietary restriction on longevity and diseases of aging in rats, have been reviewed (5, 6). Since the original 1935 Cornell reports, many repetitions of similarly designed experiments have confirmed that dietary restriction increases longevity of rodents. This work has been repeatedly reviewed (7–12). While there appears to be no difficulty in increasing longevity from restricted feeding in rodents, questions remain as to the mechanisms involved in prolonging life in this way.

(NZB/NZW)F_1, (B/W) mice (see table 1) represent one of several short-lived autoimmune-prone strains. Mice of this strain have been extensively studied as a model of human lupus erythematosus (13, 14). These mice spontaneously develop autoimmune manifestations including formation of different autoantibodies and also develop a fatal immune complex glomerulonephritis. NZB, MRL/MpJ-lpr/lpr (MRL/lpr) and male BXSB/MpJ mice are also short-lived and autoimmune-prone strains (15).

Profound influences of diet on development and expression of autoimmune disease in mice of some of these strains have previously been reported (16–22). In B/W and MRL/lpr mice, life span has been doubled

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TABLE 1

Abbreviations

List of abbreviations	
B/W	$(NZB/NZW)F_1$ New Zealand Black × New Zealand White F_1 hybrid mice
CIC	Circulating immune complexes
Con A	Concanavalin A
FCS	Fetal calf serum
HBSS	Hanks' balanced salt solution
IL-2	Interleukin-2
LPS	*Salmonella typhosa* lipopolysaccharide
MLR	Mixed lymphocyte reaction
NK	Natural killer cell
PFC	Plaque-forming cells
PHA-P	Phytohemagglutinin-P
PWM	Pokeweed mitogen
RPMI-1640	The cell culture medium used
SRBC	Sheep red blood cells
WBC	White blood cells

and sometimes even tripled by reduced calorie intake. Weindruch et al. (23, 24) have established for long-lived mouse strains that dietary restriction can be imposed as late as mid-life and still significantly increase longevity.

The present paper compares survival with dietary restriction when widely different calorie sources, as well as restricted intakes, are used. The effect of these two major dietary parameters (calorie restriction and calories source) on longevity and on a number of immunologic functions are presented. The variation in calorie intake compares "full-fed" mice with mice fed 60% of this calorie intake, and the variations in calorie source are sucrose-glycerol compared with lard as source of the nonprotein calories (70%) of the diet. It has been shown with rats that such diets are equally nutritious for weight gain and long-term maintenance even though they provide tremendous variations in calorie source. The two different diets have, however, been shown to have a profound influence on certain liver enzyme activities (25, 26).

In the present study, we have found striking differences in immunologic reactions, biochemical parameters and longevity between animals restricted in calorie intake and those unrestricted in calorie intake, whether the primary calorie source is fat or carbohydrate.

MATERIALS AND METHODS

Mice. Inbred 6-week-old female B/W mice were obtained from Jackson Laboratories, Bar Harbor, ME, and maintained in the small animal facilities of the Oklahoma Medical Research Foundation. The mice (except for one ad libitum–fed control group) were housed individually and fed as specified. Animal rooms were operated on a 12-hour light and 12-hour dark cycle. Constant temperature and humidity were maintained. Each group consisted of 15 mice.

Female CBA/H mice 2–3 months of age were used as normal animals for comparative immunologic assay, and other strains of mice were also used for Interleukin-2 (IL-2) production. All of these mice were purchased from Jackson Laboratories.

Diet. In a preliminary experiment (5 mice per group) B/W mice were fed diets in which 12, 16, 20 and 30% of the calories were protein (casein supplemented with methionine) and the rest of the calories were from sucrose-glycerol-safflower oil or from lard. Since the only group with 100% survivors to 32 weeks of age when fed ad libitum was the group fed 30% of calories as protein, 70% of calories as fat, the critical experiment reported here used this ratio. (However, groups fed ad libitum, diets with 80% and even 84% of calories as fat, and 20 or 16% of calories as protein had a few survivors through 32 weeks of age.)

A control ad libitum–fed group was given a diet containing 20% of calories as protein and 75.5% of calories as dextrin-glycerol serving as the principal energy source with 2% safflower oil (4.5% of calories) supplying the essential fatty acids. Not more than 2% of total calories are required as essential fatty acids (27). The composition of the diets is given in table 2. Glycerol is a source of carbohydrate at least equal to sucrose as an energy source and in the maintenance of glucose-6-phosphate dehydrogenase (28) and is used in the essentially fat-free diets A_1, B_1 and C to control diet dust inhalation. Note that for diets A_2 and B_2 (the high fat diets), the vitamin mixture and the mineral

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TABLE 2

Composition of diet

Constituent	A_1	A_2	B_1	B_2	C
Casein	29.4	44.12	29.4	41	19.6
Methionine	0.6	0.94	0.6	0.9	0.4
Sucrose or dextrin[1]	47.25	—	44.29	—	57.25
Glycerol	16	—	16	—	16
Safflower oil	2	—	2	—	2
AIN-76 vitamin mixture[2]	1	1.58	1.66	2.80	1
AIN-76 mineral mixture[2]	3.5	5.5	5.8	8.95	3.5
Inositol	0.05	0.08	0.05	0.08	0.05
Choline bitartrate	0.2	0.30	0.2	0.3	0.2
Lard	—	47.48	—	45.97	—
Protein kcal/ total kcal	0.303	0.308	0.310	0.308	0.202
Energy,[3] *kcal/g*	3.98	6.18	3.89	5.98	3.97

[1]Sucrose was used in diets A_1 and B_1; dextrin was used in diet C. [2]The vitamin and mineral mixes for diets A_2 and B_2 were made up in casein, whereas for diets A_1 and B_1 they were made up in sucrose. [3]Based on casein, sucrose, dextrin, glycerol, methionine, inositol, choline bitartrate calculated at 4 kcal/g and lard and safflower oil, at 9 kcal/g.

mixture were made up in casein so that these diets were carbohydrate free except for the glycerol of the fat and any excess glycogenic amino acids. Diets A_1 and A_2 were pair-fed on the basis of calories per day, while diets B_1 and B_2 were fed at 60% of the calorie intake of diets A_1 and A_2. The diets B_1 and B_2 are calculated so that feeding at 60% of the calorie intake provided equal amounts of vitamins and minerals, per animal, to those provided by the full-feeding of diets A_1 and A_2, and contained an adequate level of protein. The mice fed diet C were group fed ad libitum.

The A and B diets all contained a protein-calorie-to-total-calorie ratio of 0.30. Compared to 100 g of diet A_1 the intake of diet A_2 is 64 g to provide equal calories, whereas the intake of diet B_1 is 61.2 g and of diet B_2, 40 g, to provide 60% of the calorie intake of diets A_1 and A_2. At these ratios of food intake, diets A_1 and A_2 provide 3 g protein per 39.8 kcal while diets B_1 and B_2 provide 1.8 g protein per 23.88 kcal. At these ratios each animal was provided the same amount of vitamin and mineral mix. Diet C contains a protein-calorie-to-total-calorie ratio of 0.20 but still provides the same amounts of vitamins and minerals per calorie as diet A_1. These ratios are tabulated in table 3. Although the protein levels vary with calorie restriction, all levels are more than adequate. In addition, it has been reported earlier that differences in adequate protein intake do no affect longevity of NZB/NZW mice (17) or NZB mice, even though very low protein intake in NZB mice did influence certain immune parameters (18).

Actually, when protein intake is expressed in terms of grams per day per metabolic body mass (e.g., for body weight in kilograms$^{0.75}$), the protein intake of the small, calorie-restricted mice is in fact essentially identical to that of the much larger full-fed mice.

Representative mice were killed at 4.5 and at 6.5 months of age to permit studies of immunological function.

Cell preparation for immunological assay. Mice were bled by cutting the femoral arteries and killed by cervical dislocation. Spleens and thymuses were aseptically collected. Single-cell suspensions were prepared by gently squeezing the tissue between two glass slides in Hanks' balanced salt solution (HBSS, GIBCO Laboratories, Grand Island, NY). The suspensions were passed through a layer of gauze to remove residual large fragments. The cells were washed three times with HBSS before use.

Culture medium. RPMI-1640 medium (GIBCO) was made to 1 μM sodium pyruvate, 5 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes). It contained 100 U/ml penicillin, 100 μg/ml streptomycin,

TABLE 3

Relative amounts of nutrients and calories fed per animal per 397.6 kcals and 60% of this diet

Component	Diet and dietary intake of A_1 100 g	A_2 64 g	B_1 60 g	B_2 39 g	C 100 g
Mineral mix	3.5	3.5	3.5	3.5	3.5
Vitamin mix	1	1	1	1	1
Protein	30	30	18	18	20

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5×10^{-5} mol 2-mercaptoethanol, and 10% fetal calf serum (FCS). Normal CBA/H mouse serum (1%) was used instead of FCS for assay of mitogen stimulation and mixed lymphocyte reaction, because background stimulation is low in mouse serum compared to FCS.

Mitogen stimulation. Mitogen-induced blastogenesis was measured by incorporation of tritiated thymidine into proliferating cells. Suspensions of spleen cells were prepared in RPMI-1640 with 1% normal mouse serum. Triplicate cultures from each mouse were set up with 100 μl of 4.0×10^6 spleen cells/ml and 100 μl mitogen or medium in flat-bottomed microtiter plates (Linbro Scientific, McLean, VA). Preparations used were: phytohemagglutinin P (PHA-P, Difco Laboratories, Detroit, MI), 0.1% (vol/vol); concanavalin A (Con A, Calbiochem, LaJolla, CA), 2 μg/ml; *Salmonella typhosa* lipopolysaccharide (LPS, Difco Laboratories), 50 μg/ml; pokeweed mitogen (PWM, GIBCO), 1% (vol/vol) in RPMI-1640. The optimal concentrations of these mitogens had been previously determined and were used for stimulation in each instance. Cultures were incubated for 64 hours at 37°C in a humidified atmosphere containing 5% CO_2, then 0.4 μCi of [*methyl*-^{3}H]thymidine (specific activity 20 Ci/mmol, New England Nuclear, Boston, MA) was added for an additional 8-hour incubation. Samples were collected onto glass fiber filters by using a mini-MASH harvestor (Microbiological Associates, Walkersville, MD), then placed into vials containing ScintiLene (Fisher Scientific Co., Fair Lawn, NJ), and counted with a liquid scintillation spectrometer.

Natural killer cell (NK) activity. Spleen cells from each group were used for effector cells and YAC-1, a Maloney virus-induced T cell lymphoma originally derived from A/Sn mice, were used as target cells.

Cytotoxic assay. Cytolytic activity was tested by microcytotoxic assay. Target cells were labeled by incubating 2×10^6 cells [YAC-1] with 100 μCi sodium[^{51}Cr] chromate (Amersham, Arlington Heights, IL) for 2 hours at 37°C. Then, the cells were washed three times with HBSS and resuspended in complete medium at a concentration of 1×10^5 cells/ml. Triplicate cultures were prepared with 100 μl of labeled YAC-1 target cells. Similarly, cultures were prepared with 100 μl of spleen [effector] cells 1×10^7 cells/ml or 5×10^6 cells/ml. These two cell suspensions were used to make effector-cell-to-target-cell ratios of 100:1 and 50:1 in U-bottomed microtiter plates (Linbro Scientific). Cultures were incubated for 4 hours at 37°C in 5% CO_2, then centrifuged, and the amounts of radioactivity in the supernatants were assayed by gamma counting. The cytotoxic activity was determined by the following formula:

$$\text{\% specific cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Spontaneous ^{51}Cr release was determined by incubating target cells in an equal volume of medium only. Maximum ^{51}Cr release represents the amount released from target cells in an equal volume of Nonidet P40 detergent (Bethesda Research Laboratories, Gaithersburg, MD).

Mixed lymphocyte reaction (MLR). Cultures containing 4×10^5 responding cells and the same number of 2000 rad γ-ray irradiated allogenic stimulator cells in a total of 200 μl RPMI-1640 medium were set up in flat microtiter plates and incubated for 64 hours at 37°C in 5% CO_2, then 0.4 μCi of [^{3}H]thymidine was added for an additional 24-hour incubation, following the procedure used for the mitogen stimulation assay.

Induction of cytotoxic cells. In vitro allogenic immunization was performed in flat-bottomed, 24-well plates (Linbro Scientific). Duplicate cultures from each mouse were set up with 500 μl of 1×10^7/ml spleen cells from B/W mice and 250 μl of 2000 rad cesium-137 irradiated C57BL/6 spleen cells containing 1×10^7 cells/ml in RPMI-1640 complete medium and incubated for 4 days at 37°C in an atmosphere containing 5% CO_2. After incubation, the cultures were collected, and the cytotoxic activity of the viable cell population determined. EL-4 tumor cells, a benzo[α]pyrene-induced lymphoma of C57BL/6 origin, were used as target cells.

Induction of plaque-forming cells (PFC). The test for the in vitro PFC response to

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sheep red blood cells (SRBC) was conducted according to the method of Mishell and Dutton (29) with the following modifications.

Triplicate cultures from each mouse were set up with 500 μl of 1 × 10^7/ml spleen cells and the same volume and number of SRBC in flat-bottomed 24-well plates and incubated for 5 days at 37°C in air containing 5% CO_2. Triplicate cultures were pooled and the number of PFC determined by the method of Cunningham and Szenberg (30).

IL-2 production. Spleen cells (4 × 10^6) were suspended in 1 ml of RPMI-1640 complete medium supplemented with 2 μg/ml Con A. The cells were cultured in 24-well tissue culture plates (Linbro Scientific) for 36 hours at 37°C in an atmosphere containing 5% CO_2. Cells were removed from the culture supernatants by centrifugation at 1500 × *g* for 10 minutes. Cell-free supernatants were stored at −20°C before IL-2 assay.

IL-2 assay. The IL-2 activity of supernatants was determined by a T-cell line growth assay (31). HT-2 cells (5 × 10^3/well), BALB/c (H-2^d) SRBC-specific IL-2–dependent helper T-cell line (32), were cultured in flat-bottomed microtiter plates with the sample containing IL-2 that had been serially diluted from 1:2 to 1:64 in a total of 200 μl of RPMI-1640 medium. After 20 hours of culture at 37°C, the wells were pulsed for 4 hours with 0.4 μCi of [^{3}H]thymidine. Cultures were harvested, and radioactivity was counted in a liquid scintillation counter. The concentration of IL-2 was determined by probit analysis and one unit of IL-2 activity was defined as the amount of IL-2 containing crude culture supernate that produced 50% of the maximal proliferative response generated by the reference IL-2 preparation. Standard rat IL-2 was purchased (Collaborative Research Inc., Lexington, MA).

The IL-2 activity of supernatants was also determined by a thymocyte proliferation assay (33). In brief, supernatants were tested at final dilutions of 1:4, 1:8 and 1:16. Since the 1:4 dilution yielded the highest counts per minute and best discrimination between different samples, the results from the 1:4 dilution are presented in the figures. Cultures were supplemented with 50 mM α-methyl-D-mannoside (Sigma Chemical Co., St. Louis, Mo) to block the mitogenicity of residual Con A. CBA/H thymocytes (1 × 10^6/ml) were stimulated with PHA-P [0.1% (vol/vol)] in flat-bottomed microtiter wells in a total of 0.2 ml RPMI-1640 containing 10% FCS for 64 hours at 37°C in 5% CO_2. Then 0.4 μCi of [^{3}H]thymidine was added for an additional 8-hour incubation. Cultures were harvested and radioactivity counted.

Thymocyte response to murine IL-2. Thymocytes (1 × 10^5/well) were cultured in microtiter plates with 100 μl of a standard IL-2 preparation and PHA-P [0.1% (vol/vol)] in a total of 200 μl of RPMI-1640 medium. Cultures were incubated for 64 hours at 37°C in a humidified atmosphere containing 5% CO_2, then 0.4 μCi of [^{3}H]thymidine was added for an additional 8-hour incubation. The procedure followed was the same as used in the mitogen stimulation assay.

Assay of circulating immune complex (CIC). To measure serum levels of CIC in mice, the Raji cell radioimmunoassay as adapted for mice was used (20).

Measurement of anti-DNA antibody. Serum levels of antibodies to DNA were determined by using a modification of the Farr DNA-binding radioimmunoassay (34, 35). The results were expressed as the mean percentage of ^{125}I-labeled DNA bound by antibody and thus precipitable by ammonium sulfate. The values obtained were corrected for nonspecific precipitation in normal mouse serum.

Statistics. Statistical analyses were performed using Student's *t*-test; *P*-values <0.05 were considered significant.

RESULTS

Body and organ weights. The growth of mice fed the various diets is graphed in figure 1. The mice pair-fed diets A_1 and A_2 had a mean calorie intake of approximately 16 kcal/day. This intake of 16 kcal/day for mice weighing 40 g is identical with the maintenance energy requirement based on body weight in kilograms$^{0.75}$ as given by Canolty and Koong (36), i.e., a maintenance

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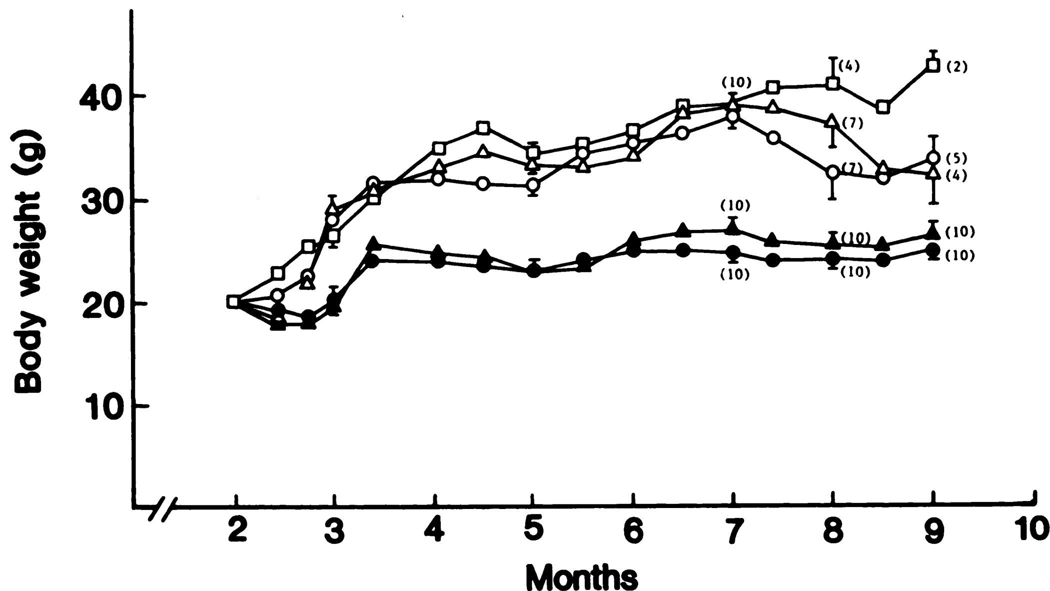

Fig. 1 Weekly average weights of the mouse groups. A_1, high sucrose diet, (○); A_2, high fat diet (△); B_1, high sucrose diet, restricted fed (●); B_2, high fat diet, restricted fed (▲); C, high dextrin diet, ad libitum fed (□). Values are means ± SEM. Numbers in parentheses represent the number surviving at that age.

requirement of 176 kcal/kg$^{0.75}$. The mice fed diet C ad libitum gained weight rapidly, reaching approximately 35 g by 4 months of age, and then gained slowly until 7 months of age. The mice restricted to 60% of the calorie intake of A_1 and A_2 (B_1, B_2, approx. 10 kcal/day) showed a slower weight gain for 4 months and then remained at this lower weight. When calorie intakes were the same, body weights were almost identical (A_1, A_2 and C; also B_1 vs. B_2). The mice of the B group fed 60% of the calories of the A group weighed approximately 70% of the weights of the A group animals. Kidney, spleen, thymus and liver weights expressed as percentage of body weight as well as actual weights are given in table 4. Actual weights of these organs were significantly decreased by dietary restriction at both ages ($P < 0.01$). However, the only consistent difference when expressed in relation to total body weight is that the liver weights of mice fed the high fat diets A_2 and B_2 are significantly lower than those of mice fed the high carbohydrate diets, presumably due to lower glycogen stores ($P < 0.05$).

As the animals in groups A_1, A_2 and C died of glomerulonephritis, their kidney weights expressed as percent of body weight increased substantially, varying from 14 to 19% of body weight as compared to the disease-free animals 5.5–7.7%.

Peripheral blood leukocytes. These data also are provided in table 4 and show higher counts in each group fed the high fat diets than in their pair-fed mates fed the high carbohydrate diets. However, animals fed the high dextrin diets also had high leukocyte counts. On both diets, restricted calorie feeding lowers the white cell count as compared to the nonrestricted feeding of the same energy source ($P < 0.05$).

Mitogen responses. In general the groups of mice with high energy and low energy intakes showed only minimal differences in mitogen responses. In figure 2 are summarized the results of the influence of diet intake and dietary composition on the responses of spleen cells to PHA, Con A, LPS and PWM. At 4.5 months of age, no differences between the groups in responses to PHA and little difference in responses to Con A were seen. However, the restricted groups (B_1, B_2) showed a higher response to

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TABLE 4

Dietary influence on body and organ weight and peripheral white blood cells (WBC)

Age	Group	Body wt	Spleen wt		Thymus wt		Liver wt		Kidney wt		WBC/mm^3
			Amount	% of body wt (× 10)	Amount	% of body wt (× 10)	Amount	% of body wt (× 10)	Amount	% of body wt (× 10)	
month		*g*	*mg*	*%*	*mg*	*%*	*mg*	*%*	*mg*	*%*	*no.*
	A_1	33.0 ± 1.6	66 ± 8	2.0 ± 0.2	65 ± 3	2.0 ± 0.1	1536 ± 84	46 ± 3	246 ± 13	7.4 ± 0.4	1210 ± 220
	A_2	36.5 ± 1.3	66 ± 12	1.8 ± 0.3	61 ± 1	1.7 ± 0.1	1456 ± 33	40 ± 1	220 ± 10	6.0 ± 0.1	2680 ± 740
4.5	B_1	24.0 ± 0.6	43 ± 12	1.8 ± 0.5	38 ± 5	1.6 ± 0.2	1110 ± 32	46 ± 2	166 ± 18	7.0 ± 0.9	780 ± 150
	B_2	25.2 ± 0.2	43 ± 8	1.7 ± 0.3	42 ± 2	1.7 ± 0.1	816 ± 110	32 ± 4	156 ± 13	6.2 ± 0.5	1640 ± 370
	C	37.5 ± 1.3	96 ± 8	2.5 ± 0.2	60 ± 3	1.6 ± 0.1	1660 ± 23	44 ± 1	203 ± 18	5.5 ± 0.6	3460 ± 430
	A_1	38.3 ± 1.0	60 ± 10	1.6 ± 0.3	70 ± 20	1.8 ± 0.3	2320 ± 130	61 ± 3	ND	ND	2940 ± 893
	A_2	39.3 ± 1.41	30 ± 10	3.3 ± 0.3	120 ± 30	3.0 ± 0.3	1900 ± 20	48 ± 1	ND	ND	4650 ± 3301
6.5	B_1	26.8 ± 0.6	45 ± 5	1.7 ± 0.2	55 ± 15	2.0 ± 0.2	1770 ± 25	66 ± 1	ND	ND	1560 ± 119
	B_2	28.9 ± 0.9	55 ± 5	1.9 ± 0.2	55 ± 5	1.9 ± 0.2	1373 ± 32	48 ± 1	ND	ND	2490 ± 270
	C	39.9 ± 0.11	30 ± 10	3.3 ± 0.3	70 ± 10	1.8 ± 0.3	2340 ± 90	59 ± 2	ND	ND	4020 ± 4801

[1]Values are means ± SEM. ND, no data.

LPS. At 6.5 months, however, the mice with the low calorie intakes showed more vigorous proliferative responses to PWM as compared to the mice with the various high calorie intakes ($P < 0.05$), whereas the differences in proliferative response to LPS seen at 4.5 months were no longer demonstrable at 6.5 months. At 6.5 months of age, no significant differences in responses to PHA-P, Con A or LPS were observed between mice in the A and B groups.

NK activity. No consistent influence of dietary intake on NK activity was observed at the time our studies were carried out in these mice (fig. 3).

MLR. In vitro proliferative responses of splenic lymphocytes from B/W mice fed the several diets were assayed against γ-irradiated allogenic C57BL/6 or CBA/H spleen cells at 4.5 and 6.5 months of age (table 5). No significant differences in response were attributable to diet at 4.5 months, but by 6.5 months the most vigorous responses were observed in the mice fed the two different diets in low amounts ($P < 0.05$). No significant difference was seen in the response to stimulator cells by the mice with either of the two low calorie intakes. Further, of the full-fed mice, differences in response were seen in the case of CBA/H stimulator cells. Mice fed the high sucrose diet appeared to respond more vigorously to CBA/H stimulator cells than mice full-fed either the high fat or high dextrin diet.

In vitro cytotoxic response to addition of allogenic antigens. The cytotoxic response of spleen cells from mice fed various diets at 6.5 months of age is presented in table 6. The results appear very similar to those obtained in studies of the MLR. The cells of mice with the low calorie intake and those full-fed the high sucrose diet responded more vigorously than mice full-fed the high fat or high dextrin diet.

In vitro PFC response to addition of SRBC. PFC responses to SRBC added to spleen cells from mice fed the several diets are recorded in table 7. It will be seen from the table that calorie restriction, regardless of dietary composition, and the full-fed diet high in sucrose and low in fat resulted in vigorous formation of antibody-producing cells, whereas the full-fed mice given the high fat or high dextrin diet showed less

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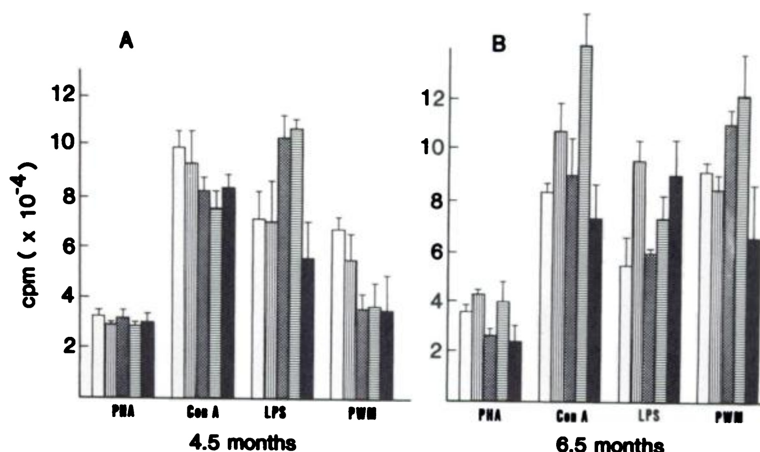

Fig. 2 Mitogen response in female B/W mice fed A_1, high sucrose diet; A_2, high fat diet; B_1, high sucrose diet, restricted fed; B_2, high fat diet, restricted fed. Bars: white, A_1; vertically striped, A_2; dotted, B_1; horizontally striped, B_2; black, C. Net [^{3}H]thymidine uptake levels after subtraction of uptake in unstimulated cultures are shown. Values are means ± SEM.

vigorous responses ($P < 0.05$). The differences were more impressive at 6.5 months of age than at 4.5 months.

IL-2 production by spleen cells. A striking biochemical abnormality of B/W autoimmune-prone mice is a deficiency in IL-2 production. This characteristic of the autoimmune-prone mice was confirmed in our studies (table 8). Spleen cells of MRL/1pr and male BXSB/MpJ, which develop severe autoimmune disease at an early stage, both showed much lower IL-2 production than mice of long-lived autoimmune-resistant strains such as DBA/2, BALB/C and CBA/H. It will be seen from table 9 that spleen cells of even young, 4.5 months old, B/W mice are unable to produce large amounts of IL-2. Mice fed a restricted calorie intake, produced at 6.5 months of age significantly more IL-2 in response to a standard stimulation with Con A in the IL-2-dependent cell line assay than did mice fed the higher calorie intakes regardless of source of calories ($P < 0.05$) (table 9). Similar observations have been made by using the mouse thymus cell proliferation assay (fig. 4). In this figure, it will be seen that improvement of IL-2 production is already noticeable in the groups with restricted intakes at 4.5 months and reached a threefold increase by 6.5 months (fig. 4).

Response of B/W thymocytes to IL-2. In table 10 are recorded observations on the responses of the thymocytes of B/W mice to stimulation with exogenous IL-2. The proliferative responses of mice with the low energy intakes were higher at both 4.5 and 6.5 months than those in mice of each of the groups fed a higher energy intake (table 10). In spite of the decrease of thymocyte response to IL-2 with age, the responses of the mice fed the restricted diets were significantly greater than those of the mice with the higher energy intake ($P < 0.05$).

Anti-DNA antibody. In table 11 are summarized data that show that the DNA-binding capacity of serum of B/W mice with

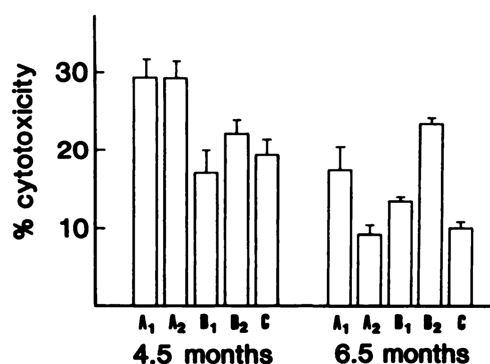

Fig. 3 NK activity of spleen cells from B/W mice fed various diets at 4.5 and 6.5 months of age. Values are means ± SEM.

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TABLE 5

The mixed lymphocyte reaction of spleen cells from B/W mice fed various diets[1]

Age	Group	Mixed lymphocyte reaction		
			Stimulator cells	
		Control	C57BL/6	CBA/H
month		*cpm*		
	A_1	4,713 ± 722	18,245 ± 1,464	ND
	A_2	5,243 ± 1,706	20,087 ± 3,123	ND
4.5	B_1	3,853 ± 352	13,784 ± 1,085	ND
	B_2	3,835 ± 165	16,560 ± 1,167	ND
	C	5,735 ± 1,466	22,528 ± 2,694	ND
	A_1	5,510 ± 1,727	37,677 ± 8,023	28,994 ± 7,480
	A_2	3,298 ± 140	16,056 ± 1,654	19,135 ± 6,252
6.5	B_1	5,448 ± 80	45,076 ± 5,827	43,434 ± 4,792
	B_2	4,219 ± 1,959	40,414 ± 5,332	42,409 ± 4,047
	C	6,289 ± 702	23,214 ± 6,542	19,799 ± 4,992

[1]Triplicate samples from three individual spleens were tested for each group. The stimulator cells from C57BL/6 or CBA/H spleen cells were irradiated with 2000 rad γ-irradiation. Values are means ± SEM; ND, no data.

a high energy intake appears greater at 4.5 and 6.5 months than the DNA-binding capacity of the serum of mice with the lower energy intake when all full-fed are compared to all restricted-fed mice ($P < 0.02$). This difference is present regardless of the composition of the diet, but the diet of higher fat composition with lower total energy intake gave the lowest serum DNA binding at 6.5 months.

CIC. CIC as measured by the Raji cell radioimmunoassay were dramatically different in mice with different calorie intakes (table 11). The mice with a low calorie intake regardless of composition had dramatically lower levels of CIC than did mice with higher energy intakes of either dietary source ($P < 0.001$). These differences were most dramatic at 6.5 months. By this parameter, there is a suggestion that the mice with the lower intake of the diet high in fat had the lowest levels of CIC. These results correlated well with the survival data (see below), since the animals with a low calorie intake regardless of dietary composition survived much longer than did mice fed a high dietary intake which had higher levels of CIC.

Survival data. Most striking of all are the survival data shown in figure 5. B/W female mice full-fed, whether the major energy source was sucrose-glycerol or lard, began to diet at approximately 7 months of age. Where there was no restriction due to pair feeding, i.e., on the control, high dextrin-glycerol diet, mortality was also rapid. On the other hand, the animals restricted in caloric intake whether from a high sucrose-glycerol or high lard diet (B_1, B_2) showed 100% survival with no evidence of glomerulonephritis in the form of proteinuria throughout the period to 12 months of age.

Urine protein is considered to be an

TABLE 6

Cytotoxic cell-mediated immune response of spleen cells from B/W mice fed various diets assayed at 6.5 months of age[1]

Group	Percentage ^{51}Cr release 5 hr	
	100:1[2]	50:1[2]
A_1	81.0 ± 2.0	70.2 ± 4.9
A_2	48.5 ± 12.2	39.0 ± 11.0
B_1	80.7 ± 2.3	74.9 ± 0.2
B_2	81.7 ± 1.9	80.2 ± 3.8
C	55.0 ± 18.7	45.2 ± 25.0

[1]Triplicate samples from three individual spleens were tested for each group. Responder spleen cells from B/W mice were stimulated in vitro with 2000 rad γ-irradiated C57BL/6 spleen cells. Chromium-51-labeled EL-4 tumor cells were used as target cells. Values are means ± SEM. [2]Ratio of effector to target cells.

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TABLE 7

In vitro PFC response at different months to SRBC in spleen cells of B/W mice fed various diets[1]

Group	PFC at 4.5 months		PFC at 6.5 months	
	per 10^6 cells	per culture	per 10^6 cells	per culture
A_1	332 ± 120	889 ± 269	657 ± 127	1553 ± 15
A_2	212 ± 50	554 ± 100	199 ± 38	379 ± 154
B_1	787 ± 131	1942 ± 381	551 ± 50	1582 ± 425
B_2	330 ± 97	868 ± 145	732 ± 300	2047 ± 1028
C	210 ± 108	413 ± 212	126 ± 50	266 ± 137

[1]Values are means ± SEM from three mice immunized 5 days earlier with SRBC.

indicator of the presence of glomerulonephropathy. From 8 months on, at all ages examined, urine protein was positive and of progressively high grade in all full-fed mice, but was negative to 12 months in the mice restricted in calorie intake.

DISCUSSION

In the present experiments, we have shown once again that in the rodent, B/W mice in this instance, low energy intake exerts a beneficial influence on longevity. The experiments reported herein were, however, designed to answer another question. We wanted to determine whether lower energy intake when it is intake of diets of greatly differing energy component and calorie density exerts similar influence on survival and development of disease in short-lived autoimmune-prone mice. Therefore, we fed diets differing greatly in that one of the diets provided 70% of calories as fat (lard) and no carbohydrate except that in the glycerol of the fat and from excesses of glycogenic amino acids in the 30% protein. The second diet provided nearly 70% of calories as sucrose plus glycerol (actual 69.5%) and only a minimum amount of fat (4.5% of calories) to provide essential fatty acids. A third diet (C) comprised of 20% of its calories as protein, 16% as glycerol, 58.5% as dextrin and 4.5% of calories as safflower oil was fed ad libitum as a normal control. Each of the diets was found to be well accepted by the mice and to produce equal weight gains quite comparable to those of mice fed laboratory diet ad libitum. Mice fed diet A_1, the high sucrose diet, were pair-fed isocalorically to the mice fed the high fat diet (A_2). In two other groups of mice, given the defined high carbohydrate and high fat diets, the calorie intake was restricted to 60% of that given the pair-fed animals. All three groups of mice fed the high calorie intake began to sicken and die between 7 and 8 months of age. They developed severe proteinuria at about this same time, and all had died by 1 year of age. By contrast, the mice fed the low calorie intake, regardless of the extremes of dietary energy source, remained well and did not have proteinurea in almost any instance until at least 14 months of age. One mouse in this group died suddenly at a year of age, whereas all the other mice in the groups maintained on restricted calories continued

TABLE 8

Interleukin-2 (IL-2) production by spleen cells from various strains of mice[1]

Group	IL-2 production 2 months
	U/ml
MLR/1pr/1pr	0.82 ± 0.03
BXSB/MpJ (male)	0.91 ± 0.37
BXSB/MpJ (female)	1.71 ± 0.06
MRL/ + +	4.21 ± 0.14
DBA/2	5.47 ± 1.12
BALB/c	6.47 ± 1.11
CBA/H	9.02 ± 0.31

[1]Spleen cells (4×10^6) were stimulated with 2 μg/ml of Con A for 36 hours. The IL-2 activity of the supernatants was determined by using a T-cell growth assay with HT-2, an IL-2–dependent cell line. Values are means ± SEM.

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TABLE 9

Effect of various diets on Interleukin-2 (IL-2) production by B/W spleen cells[1]

Group	IL-2 production	
	4.5 months	6.5 months
	U/mol	
A_1	0.78 ± 0.16	0.49 ± 0.13
A_2	0.86 ± 0.31	0.40 ± 0.03
B_1	1.12 ± 0.20	0.82 ± 0.11
B_2	0.81 ± 0.04	1.02 ± 0.24
C	0.60 ± 0.10	0.16 ± 0.15
CBA/H	15.52 ± 1.75	

[1]Spleen cells (4 × 10^6) were stimulated with 2 μg/ml of Con A for 36 hours. The IL-2 activity was determined by using a T-cell growth assay. Values are means ± SEM.

to survive in good health beyond 14 months. Thus in these experiments calorie restriction, regardless of calorie source, greatly prolonged life and inhibited development of renal disease in B/W mice. No differences were observed in survival or development of renal disease in B/W mice full-fed the higher caloric intake regardless of the dietary extremes used. It must thus be inferred that low calorie intake without other malnutrition prolongs life and delays development of disease in autoimmune-prone mice of the B/W strain.

In the case of the pair-fed animals, groups A_1 and A_2, it was always the level of consumption of high fat diet A_2 that controlled the intake of the high sucrose diet during the first 8 months. Thus, to a certain extent, three of the animals fed the high sucrose diet were protected by this dietary restriction due to pair-feeding. The animals continued to protect themselves by low food intake (probably because of developing nephritis) even after their pair-mates had died and they were fed ad libitum. The influence of pair-feeding diet A_2 on diet A_1 intake with full-feeding reflects certain immunologic differences found between the animals fed the A_2 diet and those restricted-fed the B_1 and B_2 diets and the A_1 (sucrose-glycerol) diet in MLR (table 5), cytotoxic cell-mediated immune response (table 6), and PFC response (table 7).

The influence of the pair-feeding arrangement may also be reflected in the approximations made in using the Atwater crude values of 4, 4 and 9 for the metabolizable energy value of carbohydrate, protein and fat. However, if either the 4.15, 9.4, 4.4 figures of Atwater or the 4.1, 9.3, 4.1 figures of Rubner for the metabolizable energy values of carbohydrate, fat and protein are used, the food intakes to provide equal calories (table 3) are unchanged. These may still, however, not be completely satisfactory for 1-year calculations.

As the mice in groups A_1 and A_2 died and pair-feeding was no longer possible, the mice left without pairs were then fed their diet ad libitum. For the same reason, an arbitrary figure had to be picked for groups B_1 and B_2 and they were fed 9.8 kcal per animal (weight approx. 29 g) per day (essentially their intakes as governed by 60% of A_1 and A_2), which maintained their body weights (fig. 1). This is 20% less than the maintenance requirement for a 29 g mouse of 12.3 kcal/day based on 176 kcal/$kg^{0.75}$ maintenance energy requirement (36) possibly due to feces recycling. This reflects a much greater efficiency of energy utilization by the food-restricted groups. It is,

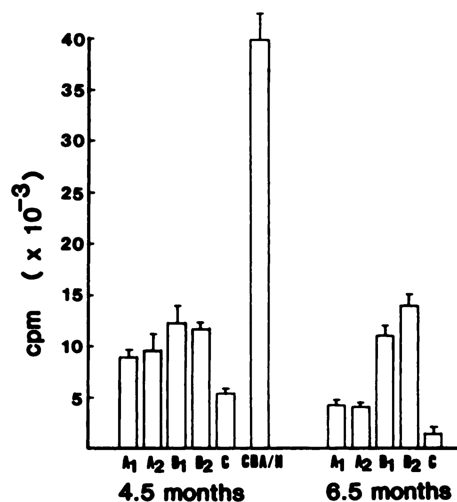

Fig. 4 IL-2 production by B/W mice with various dietary intakes. Spleen cells (4 × 10^6) were stimulated with 2 μg/ml Con A for 36 hours. The amount of IL-2 secreted into the supernatant was determined by using a thymocyte proliferation assay. Values are means ± SEM. The final dilution is 1:4.

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TABLE 10

Thymocyte proliferation in response to exogenous IL-2 in B/W mice fed various diets[1]

Group	[^{3}H]Thymidine incorporation	
	4.5 months	6.5 months
	Δcpm	
A_1	57,423 ± 2,495	18,581 ± 2,636
A_2	61,850 ± 5,976	17,259 ± 3,231
B_1	148,054 ± 24,501	25,429 ± 1,379
B_2	74,879 ± 3,725	32,300 ± 6,936
C	66,300 ± 12,883	17,129 ± 6,896
CBA/H	99,403	83,071

[1]Thymocytes from mice were stimulated with phytohemagglutinin (PHA-P) [0.1% (vol/vol)] with added IL-2. Values represent the net increase in cpm due to the addition of IL-2 to the PHA-stimulated culture. Values are means ± SEM.

however, double the basal energy requirement as calculated by the Kleiber (37) formula of 70 kcal/kg$^{0.75}$.

It appears that this increased efficiency of energy utilization, due to the experimental design and/or animal strain used, is not involved in the prolonged life span since in the careful experiment of Masoro et al. (38) efficiency of use of the energy fed was identical for maintenance of rats whether fully fed the diet according to adult weight or restricted-fed on the basis of diet and energy intake calculated according to adult weight.

It will be realized that the mice fed the lower calorie intake had lower total protein intake as well but had a protein intake per kilocalorie of diet (see table 2) and per gram body weight almost equal to that of the full-fed mice. These observations plus our earlier efforts to alter life span of B/W mice by selective protein restriction suggest that protein intake is not a critical variable. It is very clear that the lower protein content of diet C (table 3) was in no way protective (although not statistically significant, these lower protein full-fed animals appeared to be most rapidly affected by the disease).

These findings agree with and support, for mice of this strain and in a more formal way, the conclusions originally drawn by McCay et al. (1–3) that normal rats do not have shortened life spans due to richness of diet. Our findings greatly extend the range of caloric density by the use of very high fat (carbohydrate-free) diets and permit the conclusion that calorie intake and not source of calories is the most critical dietary variable in development of disease and shortness of life in mice of this strain.

There are many reports of effects of varying dietary fat levels on immunological function and the development of autoimmune disease as well as the incidence of tumors (39, 40). Levy et al. reported fat effects on the immune response, production of anti-viral factors and immune complex disease in B/W mice (40), whereas Kelly and Izui reported that fat accelerates lupus nephritis (39). In both cases their animals were, however, full-fed; and it can be seen from the present report that total calorie intake overrides effects of source of calories. In the current experiment the increase in caloric density of the high fat diet was 50% over that of the high carbohydrate diet,

TABLE 11

Dietary influence on serum anti-DNA antibody and circulating immune complex (CIC) in female B/W mice[1]

Group	% DNA binding		CIC[2]	
	4.5 months	6.5 months	4.5 months	6.5 months
	%		*μg/ml*	
A_1	21.3 ± 3.3	29.2 ± 9.9	309 ± 47	610 ± 32
A_2	35.8 ± 6.6	44.9 ± 1.7	330 ± 69	1168 ± 16
B_1	15.3 ± 5.6	24.0 ± 1.3	13 ± 3	152 ± 60
B_2	15.2 ± 7.8	11.0 ± 3.3	27 ± 10	40 ± 10
C	23.5 ± 0.9	43.4 ± 8.2	656 ± 93	1048 ± 100

[1]Each group consisted of three mice. Values are means ± SEM. [2]Results are expressed as microgram equivalents of aggregated murine IgG per milliliter of murine serum.

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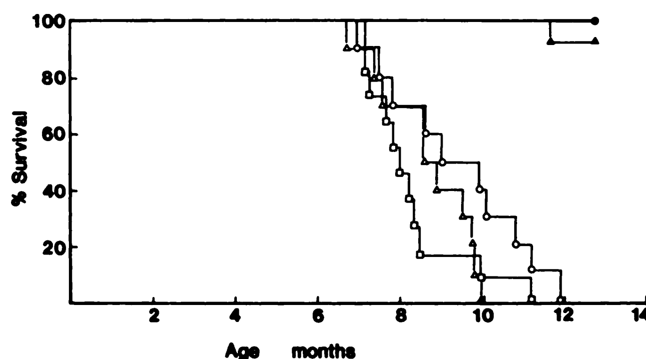

Fig. 5 Survival data for female B/W mice fed A_1 (○), A_2 (△), B_1 (●), B_2 (▲), or C (□) diets.

while in the Levy et al. experiment (40), the increase in caloric density due to fat addition was only about 20%.

Walford has stated that decreased caloric intake (energy intake) without malnutrition may influence greatly life span of many species from fish to man (41).

Our observations also confirm and place in a new perspective the earlier findings of Fernandes et al. (16–18), which revealed the extraordinary influence of diet on autoimmune-prone strains of mice. As was demonstrated by Fernandes et al. (16), dietary intake was shown in the present studies to have a dramatic influence on certain immunoparameters as well. The influence on certain of these parameters in the present study correlates well with the dietary influence on development of major disease manifestations and longevity, while other changes of immunoparameters were less well correlated with prevention of disease and prolongation of life in this model system. For example, it was shown in the present studies that low calorie intake, regardless of extreme variations in dietary composition, interfered with development of CIC and also decreased production of anti-DNA antibody (table 11), increased IL-2 responses of thymocytes (table 10), increased proliferative responses to PWM at 6.5 months (fig. 2), increased capacity of spleen cells for IL-2 production at 6.5 months (table 9) and apparently increased proliferative response in MLR (table 5) while preventing development of renal disease and greatly promoting longevity.

Dietary restriction, regardless of dietary composition, increased PFC response to SRBC in vitro (table 7), enhanced the vigor of cell-mediated cytotoxicity development in vitro (table 6) and increased the MLR of spleen cells to C57BL/6 stimulator cells at 6.5 months (table 5). The latter three influences on immunological parameters were also seen in B/W mice full-fed the low fat, high sucrose diet. The low fat, high sucrose diet that exerted these latter influences on immune function, however, did not prevent renal disease or promote longevity in the mice. It will be necessary to investigate this influence further to explain the rather striking difference between the two low fat diets used (sucrose vs. dextrin). This difference may be related to rate of energy absorption with restricted intake or sucrose being more rapid and/or more complete than fat or dextrin when these diets are full fed.

The production of IL-2 by Con A–stimulated spleen cells was shown by Dauphinée et al. (33) and Altman et al. (42) to be very low in B/W mice and in mice of other autoimmune-prone strains. This immunologic abnormality, indeed, is one of the few abnormalities present extremely early in life in B/W mice. Thus, it seemed most challenging that restriction of energy intake restored IL-2 production significantly. Whether the restoration that occurred is of major biological significance remains questionable, however, because the animals with low calorie intake were restored only to a level of IL-2 formation that remains as much as 5 times lower than that seen in many strains of mice

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that are resistant to development of autoimmunities.

The findings reported herein make it extremely important, whenever pharmacological manipulations or other maneuvers prolong life, delay onset of renal disease, decrease CIC formation, inhibit anti-DNA antibody production or influence other immunological or hematological parameters of autoimmune-prone mice, that careful attention be paid to the influence of the manipulation in question on dietary and particularly on energy intake of the mice.

Other dietary or pharmacological manipulations, that have fundamental influences, perhaps similar, perhaps different, than those imposed in our study have also been shown to affect survival, development of renal disease, liver polyploidy (43), autoimmunities and immunologic function, in B/W and other autoimmune-prone mice as well as in normal mice, rats, or other animals.

Dietary restrictions reported to result in prolongation of life include low intake of phenylalanine-tyrosine (44, 45), zinc (46), essential fatty acids (47) or calories (all of which result in smaller animals). The "life-prolonging compounds" that have been administered include prostaglandin E_1 (48), eicosapentaenoic acid (49) and actinomycin D (50). It seems possible that some of these manipulations that result in prolongation of life, operate through mechanisms different from those where calorie restriction is the sole critical dietary variable. But from the present observations, it seems crucial in all studies to record intake of total calories and other food components consumed as well as weights of the animals.

The fundamental mechanism underlying the profound influence of calorie undernutrition without malnutrition in autoimmune-prone as well as long-lived strains of mice still must be discovered. Perhaps Gabrielsen (50) has discovered a crucial variable in her view of the pathogenesis of disease in B/W mice as an abnormality of DNA metabolism or turnover. As she states, however, the calorie intake of the actinomycin D–treated mice was decreased, as regularly occurs with actinomycin D feeding.

In the earliest experiments of our series, Fernandes et al. (17, 51, 52) reported influences of dietary composition on longevity and disease development in NZB mice. However, those experiments, while they launched an extensive series of studies, were done with diets the exact composition of which could not be determined and in which exact measurements of food intake of the individual mice were not made. Prickett et al. (49) have more recently presented evidence that dietary composition of a particular sort can inhibit disease development, anti-DNA production and prolong life in B/W mice. These observations are not in conflict with our own, but both studies require an understanding of the fundamental changes that underlie the striking influence of diet on longevity and pathogenesis of disease in these mice.

Masoro (37) carried out studies in normal rats which led him to conclude that the influence of dietary restriction on longevity of these animals was not attributable to changes in metabolic rate as has been claimed. It would be our contention that in the model system we are studying, where calorie restriction of 40% can double and sometimes triple life span, that this issue would have to be settled by direct analyses of metabolism and the biochemistry of energy-linked processes, particularly since length of life can be so greatly prolonged by calorie restriction. In this system, it is certain that lifetime calorie intake and metabolism are significantly increased in our restricted animals.

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