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Immunology

Influence of early or late dietary restriction on life span and immunological parameters in MRL/Mp-lpr/lpr mice

(nutrition/autoimmunity/calories/longevity)

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ABSTRACT **Reduced food intake doubles and even triples the life span of (NZB × NZW)F_1 (B/W) mice and greatly influences immunological function. Here we investigate the influence of food intake while keeping vitamin and mineral intake constant in mice of the MRL/Mp-lpr/lpr (MRL/l) strain. Restriction of food intake greatly prolongs life. This influence also was seen when dietary restriction was imposed later in life. Dietary restriction inhibited development of lymphoproliferative disease and greatly decreased the numbers of cells in thymus, lymph nodes, and spleen. It also delayed development of glomerulonephritis and maintained certain immunological responses. Proliferative responses to phytohemagglutinin, pokeweed mitogen, or allogeneic spleen cells were maintained in the mice fed a low-calorie diet from 6 wk. Imposing diet at 12 wk had a lesser influence than earlier restriction. These dietary influences did not depress formation of anti-DNA antibodies or circulating immunocomplexes. MRL/l mice show an apparently extremely low production of interleukin 2, and dietary restriction increased the capacity of lymph node cells but not spleen cells to produce this immunomodulator.**

Dietary manipulations produce striking influences on immunologic functions, disease manifestations, and longevity in autoimmune-prone mice (1–5). Protein restriction affected immunologic function but not disease in NZB mice (3). Total food restriction in (NZB × NZW)F_1 (B/W) mice produced increased longevity (4), preservation of certain immunologic functions, and inhibition of development of glomerulonephritis (5), of anti-DNA and anti-glycoprotein 70 (gp70) antibody production (6), and of circulating immunocomplex (CIC) formation (7). Delay of dietary imposition also inhibited progression of renal lesions (8). In recent experiments, reduced intake of a high carbohydrate/2% fat diet or reduced intake of an extremely high fat/no carbohydrate diet each promoted longevity, decreased immunopathology, and influenced immunoparameters of B/W mice (9). We report herein influences of food restriction on longevity, development of renal and lymphoproliferative disease, and immunologic function in another autoimmune-prone strain of mice, MRL/Mp-lpr/lpr (MRL/l). Later, as opposed to earlier, imposition of the restricted dietary intake showed that food restriction, imposed earlier or later, greatly influenced life span, development of lymphoproliferative and renal diseases, and maintained certain immune functions in autoimmune-prone MRL/l mouse.

MATERIALS AND METHODS

Mice. Inbred 6-wk-old male MRL/l, MRL/Mp-+/+ (MRL/n), and C57BL/6 mice were obtained from The Jackson Laboratory and maintained in the small animal facilities of the Oklahoma Medical Research Foundation. MRL/l mice were housed individually and fed as specified. Animal rooms had a 12-hr light/12-hr dark cycle and constant temperature and humidity. Each group comprised 14 or 16 mice. MRL/n and C57BL/6 mice were housed in groups and fed on lab chow diet ad lib.

Diets. Diets were constructed according to methods previously described (4). Composition was 22% casein, 33% dextrose, 33% starch, 5% corn oil, 4% mineral mixture, 2% vitamin mixture, and 1% agar. Diets were prepared every week and stored at 4°C. A fixed amount of food, 20 cal/day, was given to groups on "normal" intake, and exactly half that (10 cal/day per mouse) was provided to the mice on restricted calorie intake. Calorie-restricted mice were given vitamin and salt mixture equal to that for mice on the normal diets. Restriction was initiated at age 6 wk. After 6 wk on a high- or low-calorie intake, half of either group were switched to the opposite regimen. Mice on lower calorie intake were placed in the higher calorie intake group and mice fed the higher calorie intake were fed the lower calorie intake.

Representatives were killed at ages 3 and 5 mo to permit immunological analyses.

Culture Medium. RPMI-1640 medium (GIBCO) was made 1 μM in sodium pyruvate, and 5 mM in Hepes; it contained 100 units of penicillin per ml, 100 μg of streptomycin per ml, 50 μM 2-mercaptoethanol, and 10% fetal calf serum. Normal CBA/H mouse serum (1%) was used instead of fetal calf serum for assay of mitogen stimulation and mixed lymphocyte reaction.

Mitogen Stimulation. Mitogen-induced blastogenesis was measured by a method previously described (9). Preparations used were: phytohemagglutinin P (PHA; Difco), 0.1% (vol/vol); concanavalin A (Con A; Calbiochem), 2 μg/ml; *Salmonella typhosa* lipopolysaccharide (LPS; Difco), 50 μg/ml; and pokeweed mitogen (PWM, GIBCO), 1% (vol/vol) in RPMI-1640 medium.

Natural killer cell activity, induction of cytotoxic cells, mixed lymphocyte reaction assay and induction of plaque-forming cells with sheep erythrocytes (SRBC) was measured as described earlier (9).

Interleukin 2 (IL-2) Production. Spleen cells (2×10^6) were suspended in 1 ml of RPMI-1640 complete medium supplemented with 2 μg of Con A per ml. The cells were cultured in 24-well tissue culture plates (Linbro) for 36 hr at 37°C in an atmosphere containing 5% CO_2. Cells were removed from the culture supernatants by centrifugation at 1500 × *g* for 10 min. Cell-free supernatants were stored at −20°C until the IL-2 assay was carried out.

IL-2 Assay. The IL-2 activity of supernatants was determined by quantifying the influence of the supernatants on

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Abbreviations: B/W mice, (NZB × NZW)F_1 mice; CIC, circulating immunocomplex; Con A, concanavalin A; IL-2, interleukin 2; LPS, *Salmonella typhosa* lipopolysaccharide; MRL/l mice, MRL/Mp-lpr/lpr mice; MRL/n mice, MRL/Mp-+/+ mice; PHA, phytohemagglutinin P; PWM, pokeweed mitogen; SRBC, sheep erythrocytes; ss DNA, single stranded DNA; gp, glycoprotein.

growth of IL-2-dependent T-cell line (10). HT-2 cells (5×10^3 cells per well), a BALB/c ($H\text{-}2^d$) SRBC-specific IL-2-dependent helper T-cell line (11), were used.

The IL-2 activity of supernatants was also determined by a thymocyte proliferative assay (9, 12).

Assay of CIC. To measure serum levels of CIC in mice, the Raji cell radioimmunoassay as adapted for mice was used (7). Results are expressed as μg equivalents of aggregated murine IgG per ml of serum.

Measurement of Anti-Single-Stranded (ss) DNA Antibody. Serum levels of antibodies to ss DNA were determined by using a modification of the Farr DNA-binding RIA (13). The results were expressed as the mean percentage of ^{125}I-labeled ss DNA bound by antibody and, thus, precipitable by ammonium sulfate. The values obtained were corrected for nonspecific precipitation in normal mouse serum.

Proteinuria. Proteinuria was assayed with tetrabromphenol paper (Combistix; Ames, Elkhart, IN) on fresh urine sample. The test is graded 1–4$^+$ (1$^+$, <30 mg/100 ml; 2$^+$, <100 mg/100 ml; 3$^+$, <300 mg/100 ml; and 4$^+$, >2000 mg/100 ml). In this experiment, high-grade proteinuria was designated as $\geq 2^+$.

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

RESULTS

Growth Curve. Mice were of four groups. Group I had a high-calorie diet (20 cal/day per mouse) throughout (designated H/H); group II, high-calorie diet between 6 and 12 wk and low-calorie diet (10 cal/day per mouse) after 12 wk (H/L); group III, low-calorie diet from 6 to 12 wk and a high-calorie diet thereafter (L/H); and group IV, low-calorie diet throughout (L/L). Growth of mice on the four diets is shown in Fig. 1.

Mice fed on high calories gained weight rapidly, reaching approximately 50 g by 3 mo. They lost weight after 4 mo of age. Mice fed the low-calorie diet throughout (group IV) showed little change in weight. They lost a small amount of weight at first and then kept constant for a prolonged period. Mice of group IV (L/L) weighed approximately 54% of mice of group I (H/H). Mice fed a high-calorie diet until 12 wk and then a low-calorie diet thereafter (group II) (H/L) lost weight rapidly from a maximum of 50 g at 12 wk to 27 g 6 wk after restriction and then kept a constant weight thereafter. They were only slightly larger than those mice on low-calorie intake throughout. Mice of group III (L/H) fed a low-calorie diet for 6 wk then a high-calorie diet after age 12 wk gained weight rapidly on the high-calorie diet. However, their weight was a bit lower than those fed high calories throughout.

Longevity of MRL/l Mice Fed Different Diets. Fig. 2 summarizes data on the life span of MRL/l mice fed different diets. Mice fed the L/L diet had the longest life span. Mice fed the H/H diet had the shortest life span (218 ± 18 days). Mice fed the L/H diet were also short-lived (247 ± 29 days), and life span was not different from that of MRL/l mice fed the H/H diet. Mice fed H/L diet had an intermediate life span. Median survival on the H/H and L/H diets was 7 mo. Median survival on the H/L diet was 15 mo and that of the L/L was not determinant. Life span of the calorie-restricted mice was more than doubled even when calorie restriction was delayed until the onset of disease in MRL/l mice.

Cell Numbers. Absolute numbers of cells in spleen, thymus, axillary lymph node, and peripheral blood were all affected significantly by dietary restriction (Table 1). The animals fed the H/H diet had enlarged spleen, thymus, and axillary lymph nodes. This reflected the lymphoproliferative disease in these mice, which is manifest by age 3 mo. They showed the highest number of spleen cells, thymus cells, and axillary lymph nodes cells at 12 wk. Mice on the low-calorie diet throughout had fewer spleen cells, thymus cells, axillary lymph node cells, and peripheral blood cells, which were proportionately smaller than body weights ($P < 0.05$). At 20 wk, these differences were even more striking. Spleen cell numbers of mice on the H/H diet were nearly 4 times the spleen cell number of mice on the L/L diet, and axillary lymph node cells were approximately 10 times higher. The enlargement of spleen and axillary lymph node in H/L or L/H groups were intermediate between these two groups. Thymuses of mice fed the H/L diet were smaller than the thymuses of the mice fed the L/H diet ($P < 0.01$). Those fed the L/H diet had cell numbers equal to those on the H/H diet. The thymuses of the H/L and L/L groups were unusually small as compared to the thymuses of other strains—e.g., CBA/H. Circulating leukocyte counts were lower on the L/L diets at 12 wk than on the H/H diets. At 20 wk, the mice fed the H/H diet had the highest leukocyte counts; mice fed the L/L diet, the lowest leukocyte counts; and mice fed H/L and L/H diets, intermediate leukocyte counts.

Proteinuria in MRL/l Mice on the Different Diets. All mice fed the H/H diet developed severe proteinuria by age 6 mo (Fig. 3) and began to die. Mice fed the L/L diet had a low incidence of proteinuria by age 1 yr. The mice fed the L/H diet also developed proteinuria sooner than mice fed the L/L

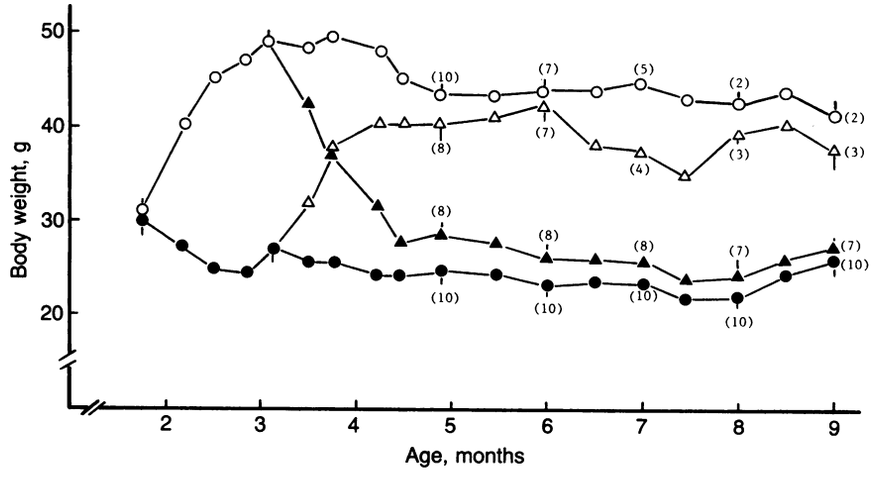

FIG. 1. Body weights of MRL/l mice with different calorie intakes. ○, High-calorie diet throughout (H/H); ▲, high-calorie diet between age 6 and 12 wk and a low-calorie diet after 12 wk (H/L); △, low-calorie diet from 6 to 12 wk and a high calorie diet thereafter (L/H); ●, low calorie diet throughout (L/L). Values are means ± SEM. Numbers in parentheses are the number surviving at that age.

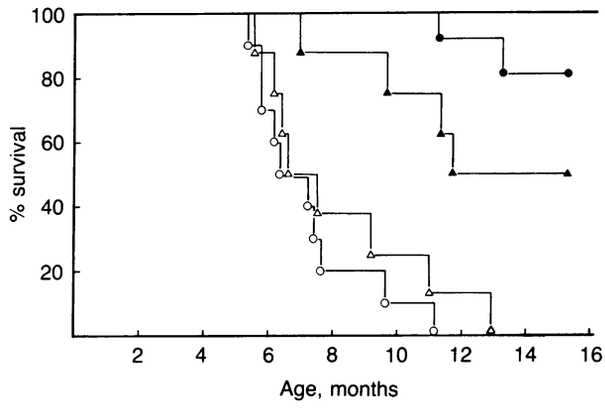

FIG. 2. Survival data of MRL/l mice fed on H/H (○), H/L (▲), L/H (△), or L/L (●) diet.

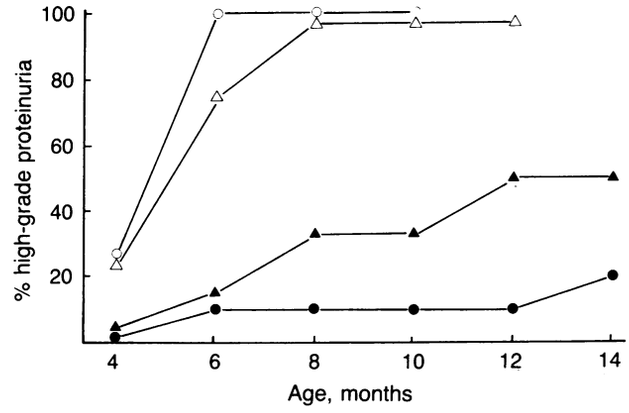

FIG. 3. Effect of calorie intake on the cumulative progression to high-grade proteinuria (≥100 mg/ml) up to age 14 mo.

or H/L diet. The mice fed the H/L diet had a lower incidence of proteinuria than did mice fed the H/H or L/H diets.

Immune Parameters. *Mitogen responses.* Influence of calorie intake on the responses of spleen cells to PHA, Con A, LPS, and PWM is shown in Fig. 4. At 12 wk, the mice fed the low-calorie diet showed more vigorous proliferative responses to PHA and PWM than did mice with high-calorie intake ($P < 0.05$). This difference was also demonstrated at 20 wk. The mice fed low calories after 12 wk, group II (H/L), had higher responses to PHA or PWM in spleen than did mice fed the H/H diets ($P < 0.05$). Mice with high-calorie intake after age 12 wk, group III (L/H), showed poor responses to PHA and PWM in spleen compared with mice fed the L/L or H/L diet ($P < 0.05$). No differences were seen between the groups in response to Con A or LPS at either ages 12 or 20 wk.

Mixed lymphocyte reaction. Differences were observed in proliferative responses of lymphocytes to allogeneic cells in mice fed high- or low-calorie diets (Table 2). The spleen and lymph node cells of MRL/l mice fed low-calorie diets (L/L) responded more vigorously to allogeneic C57BL/6 stimulator cells than did mice fed high-calorie diets (H/H) at both ages 12 and 20 wk ($P < 0.05$). In mice fed the H/L diet, responses of spleen cells improved by age 20 wk, and when calorie intake was changed from low to high after age 12 wk (L/H), responses of lymph node cells decreased compared with that of animals fed low-calorie diets ($P < 0.05$).

IL-2 production by spleen or lymph node cells. A biochemical abnormality reported in autoimmune-prone mice is deficiency of IL-2 production. In MRL/l mice, spleen cells of even young mice age 2 mo were unable to produce even 1 unit of IL-2 per ml in the IL-2-dependent cell line assay (data not shown).

IL-2 activity of supernatants from cultured cells also was determined by using the somewhat more sensitive but less discriminatory mouse thymus cell proliferation assay (Table 3). A deficiency of IL-2 production of spleen cells was present in all groups at both ages 12 and 20 wk, and no differences were attributable to diet. IL-2 production by lymph node cells was higher in mice fed low calories than in mice fed high calories at age 12 wk. At 20 wk, a rather striking increase of IL-2 production by lymph node cells was demonstrable in the mice fed the L/L diet as compared with those on the H/H diet ($P < 0.05$). The levels of IL-2 production observed in the mice with low-calorie intake from age 6 wk were comparable to levels of IL-2 production observed with spleen cells in MRL/n mice but still much lower than those observed in autoimmune-resistant strains.

Dietary influence on anti-ss DNA antibody and CIC levels. Table 4 compares influence of diet on anti-ss DNA antibody titers and CIC levels in MRL/l mice. MRL/l mice have striking increases in levels of anti-ss DNA antibodies and CIC. In analyses carried out on mice at both ages 12 and 20 wk, none of the dietary regimens affected significantly either anti-ss DNA antibody levels or the levels of CIC. Finally, studies were carried out by measuring CIC concentration in the long-surviving mice on L/L and H/L diets at age 1 yr. High levels of CIC (>3600 μg/ml) were found in all of these long-surviving MRL/l mice. In spite of these high levels of CIC, the mice continued to thrive.

Although natural killer cell activity, specific cytotoxic T-cell function, and response of plaque-forming cells toward SRBC were studied, and even though significant influences were demonstrated by restriction of dietary intake at 12 wk, no consistent or persistent influence of the restricted diet was seen at age 20 wk that could have influenced the longevity of the mice (data not shown).

DISCUSSION

MRL/l mice represent an autoimmune-prone strain. Both males and females develop lymphoproliferative diseases, autoimmunity, and rapidly progressive renal disease. Disease appears in these mice as early as age 2–3 mo, and all animals die by age 8–10 mo (14, 15). Median survival time of these mice is approximately 6 mo for males and 5 mo for

Table 1. Dietary influence on cell number of organ and peripheral blood in MRL/1 mice

Age, wk	Group	Body weight, g	Organ cells × 10^{-7}			Peripheral leukocytes per mm^3
			Spleen	Thymus	Axillary lymph node	
12	H	50.4 ± 1.0	18.4 ± 0.4	16.6 ± 3.0	13.5 ± 1.9	4620 ± 420
	L	27.0 ± 1.3	5.8 ± 1.0	7.0 ± 2.0	3.5 ± 0.7	3150 ± 200
20	H/H	45.0 ± 0.9	49.0 ± 10.9	7.8 ± 2.3	76.0 ± 7.9	7770 ± 2850
	H/L	27.4 ± 2.8	16.4 ± 6.8	0.8 ± 0.1	35.6 ± 19.6	4320 ± 209
	L/H	40.2 ± 0.8	22.8 ± 4.8	8.8 ± 1.4	36.6 ± 9.0	3510 ± 870
	L/L	24.1 ± 0.7	13.2 ± 7.5	0.8 ± 0.2	7.8 ± 5.8	1290 ± 510

Values are means ± SEM.

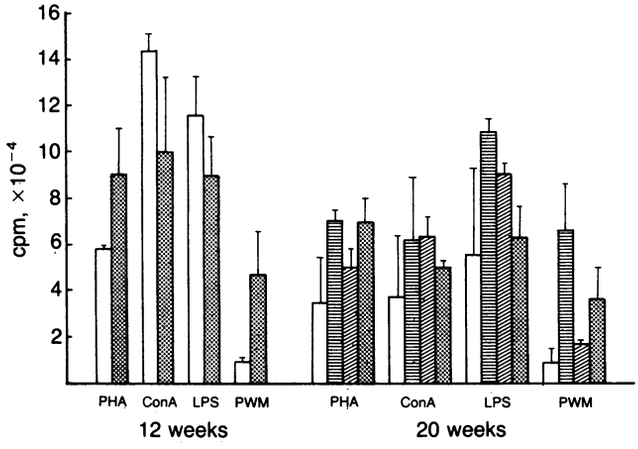

FIG. 4. Mitogen responses of spleen cells from MRL/l mice at ages 12 and 20 wk fed H/H (▭), H/L (▬), L/H (▨), or L/L (▩) diet. Net [^{3}H]thymidine uptake levels after subtraction of uptake in unstimulated culture are shown. Values are mean cpm ± SEM.

females. In prior studies with influence of diet on mice of the MRL/l strain, restriction of calories inhibited development of the lymphoproliferative disease and immunocomplex-based renal disease and spared early involution of certain immunological functions (16). Studies of mice of the MRL/l strain was remindful of B/W autoimmune-prone mice in which diet restriction greatly prolonged life, inhibited development of autoimmunity, and prevented decline of immunological functions (9).

In the present experiments, total food and calorie restriction imposed at age 6 wk and calorie restriction imposed at 12 wk greatly prolonged median survival time, inhibited development of proteinurea, prevented development of lymphoproliferative disorder, and also prevented development of thymic lymphoid lesions and cell accumulation in spleen and lymph nodes. Decrease of cell numbers in spleen and lymph nodes was affected by dietary restriction in the present experiment. By contrast, early food restriction imposed between ages 6 and 12 wk, when followed by a higher food intake for the remainder of life, did not prolong life nor inhibit early development of renal disease or lymphoproliferative disease.

Restriction of food intake was accompanied by maintenance of proliferative responses to stimulation of lymphoid cells with PHA, PWM, or allogeneic cells. A deficiency of

Table 2. The effects of dietary restriction on the mixed lymphocyte reaction in MRL mice*

Organ	Age, wk	Group	Control	C57BL/6 stimulator cells
Spleen	12	H	3039 ± 233	11,074 ± 120
		L	5575 ± 188	21,730 ± 14
	20	H/H	3097 ± 48	5167 ± 1412
		H/L	2753 ± 1039	17,195 ± 1642
		L/H	3752 ± 466	5967 ± 1307
		L/L	4637 ± 636	21,374 ± 13,886
		MRL/n	3268 ± 633	45,604 ± 7512
Lymph node	12	H	1163 ± 531	7525 ± 652
		L	2869 ± 838	42,606 ± 10,628
	20	H/H	1257 ± 35	2312 ± 934
		H/L	1114 ± 682	4557 ± 2823
		L/H	2111 ± 277	4151 ± 682
		L/L	2037 ± 60	21,594 ± 8445

Values are mean cpm ± SEM.
*Cell suspensions were incubated for 64 hr at 37°C, and then [^{3}H]thymidine was added for an additional 24 hr incubation.

Table 3. Dietary influence on IL-2 production by spleen or lymph node cells of MRL mice*

Organ	Age, wk	Group	[^{3}H]Thymidine incorporation†
Spleen	12	H	833 ± 88
		L	699 ± 99
	20	H/H	937 ± 540
		H/L	2298 ± 1658
		L/H	1149 ± 467
		L/L	1579 ± 417
		MRL/n	13,190 ± 5024
Lymph node	12	H	3200 ± 349
		L	8420 ± 2954
	20	H/H	2493 ± 275
		H/L	6491 ± 2758
		L/H	7966 ± 1816
		L/L	11,124 ± 5022

*The cells of spleen or lymph node (2×10^6 cells per ml) were stimulated with 2 μg of Con A per ml for 36 hr.
†The amount of IL-2 secreted into the supernatant was determined by using a thymocyte proliferation assay. Values are mean cpm ± SEM. A final dilution of 1:4 was used.

IL-2 production present in both spleen and lymph node cells was significantly corrected, especially for the lymph node populations, to levels characteristic of the MRL/n mice. However, even these levels are lower than in certain autoimmune-resistant mice—e.g., CBA/H (9). Not so much affected by the dietary restriction was antibody formation *in vitro* to stimulation with SRBC, cytotoxicity to allogeneic stimulation (data not shown), and proliferative responses of spleen cells to Con A or LPS. Further, proliferative responses of thymus cells to stimulation with IL-2 were also not much affected by the dietary restriction imposed (data not shown).

Rather surprising to us was the finding in these studies that in spite of the great prolongation of life and delay in the development of renal disease, neither antibody production to ss DNA or the formation of CIC were decreased significantly.

A most important finding was that dietary restriction could be imposed as late as age 12 wk and after disease manifestations had already appeared in mice of the MRL/l strain. Such late restriction of diet, like early dietary restriction, influenced very dramatically the length of survival, development of renal disease, and certain immunological parameters in these mice. These findings are consonant with those of Friend *et al.* (8) in B/W mice and agree with implications of the studies of Weindruch *et al.* (17) for the longer-lived mice. The exact mechanisms of prevention of the lymphoproliferative disease and renal disease in mice of this strain remain enigmatic, especially since neither anti-DNA nor antigen–antibody complex formation was reduced by the dietary restriction. Only future analyses will tell whether, in mice of this strain, as in B/W mice, dietary restriction exerts important suppressive action on production of retroviral gp70–

Table 4. Dietary influence on serum anti-ss DNA antibody and CIC in MRL mice

Age, wk	Group	% DNA binding	CIC,* μg/ml
12	H	39.0 ± 7.5	3420 ± 1360
	L	39.7 ± 6.0	2840 ± 400
20	H/H	67.6 ± 7.9	>3600
	H/L	58.6 ± 3.6	>3600
	L/H	62.1 ± 10.2	>3600
	L/L	52.7 ± 7.1	>3600
	MRL/n	23.1 ± 0.7	920 ± 520

*Results are expressed as μg equivalents of aggregated murine IgG per ml of murine serum. Values are means ± SEM.

anti-gp70 antigen–antibody complexes, which the studies of Izui *et al.* (18) have suggested may be especially important in pathogenesis of renal and vascular disease in both B/W and MRL/l mice.

Essential future studies will determine whether the restriction alters coupling of energy metabolism (19), influences cell cycles, and alters the rate of cell proliferation or cell turnover in critical sites as might be implied by the studies of Gabrielsen *et al.* (20). Perhaps an influence on Ia antigen expression, which can be achieved in another way by administering monoclonal antibodies against Ia antigens (21), or influences on the radiosensitive stem cells, which contain the potential for development of disease in certain autoimmune-prone mice (e.g., NZB mice), might help to explain our findings. Two findings deserve special attention. Diet-restricted animals, which lived long lives, had a significant correction of deficient IL-2 production, especially in their lymph node cells. This seems particularly important because it has been reported that autoimmune-prone mice characteristically have very deficient production of IL-2 (12, 22); however, this characteristic has been disputed and attributed to dilution of IL-2-producing cells in the spleen of the autoimmune-prone mice (23). Further, the diet-restricted mice showed a decrease in thymus size, and this influence of diet may have exerted some of the influence that thymectomy early in life exercises in mice of these strains (24–26).

Either early or later dietary restriction inhibits development of the genetically determined lymphoproliferative disease and the rapidly progressive renal disease without significantly suppressing anti-DNA production or greatly limiting CIC formation. Our investigations show dramatic influences of diet in animals of each of the autoimmune-prone strains studied thus far. The burden of future investigations must be to extract the mechanisms of these powerful influences of the experimental diets on life span and development of disease.

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