Effects of Nutrition on Disease and Life Span

I. Immune Responses, Cardiovascular Pathology, and Life Span in MRL Mice

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Mice of the autoimmune, lymphoproliferative strain MRL/lpr and the congenic, nonlymphoproliferative strain MRL/n were fed one of six diets from weaning onward. These mice were sacrificed at 3 or 5 months of age. Low fat diets resulted in lower cholesterol and higher triglyceride levels than did cholesterol-containing high-fat diets. Caloric restriction of MRL/lpr mice was associated with an increased plaque-forming cell response to trinitrophenylated polyacrylamide beads, less lymphoproliferation, and less severe glomerulonephritis. Diet did not affect the incidence of autoimmune vasculitis in MRL/lpr mice sacrificed at 5 months. MRL/lpr mice fed a low-fat, calorically restricted diet from 5 months of age to death lived longer than mice which were fed *ad libitum* a cholesterol-containing, high-fat diet. At death, MRL/lpr mice fed the former diet had the autoimmune vasculitis which had been evident in mice killed at 5 months, whereas mice fed the latter diet, in addition to the vasculitis, had a high incidence of atherosclerotic lesions of intrarenal and aortic branch arteries. (Am J Pathol 1984, 117:110–124)

THE MRL MOUSE strain is divided into two congenic substrains: MRL/MPJ-lpr/lpr (MRL/l) and MRL/MPJ-+/+ (MRL/n), which differ with respect to the presence of the lpr (lymphoproliferative) gene. Animals carrying the lpr gene develop massive proliferation of T cells and die between 5 and 7 months of age. Pathologic study of MRL/l mice at death reveals glomerulonephritis, myocardial infarction, and arteritis. MRL/n mice have greater longevity, with life expectancy of 17 to 23 months. Neonatal thymectomy prevents the appearance of the lymphoproliferative disease in the MRL/l mice. Bone marrow cells from MRL/l mice are accepted by immunologically competent MRL/n recipients and transfer all aspects of the disease.[1–4] Thus, the two substrains offer a uniquely controlled model for investigation of the effects of diet on autoimmune and vascular disease.

Forty years ago McCay et al showed that caloric restriction increased the longevity of male rats.[5] Rats with restricted access to food have been reported to weigh less and have less severe proteinuria, lower blood urea nitrogen (BUN) concentrations, less severe renal disease, and longer life spans.[6] Subsequently, it was shown that rats that voluntarily ate less than their littermates weighed less and lived longer.[7,8]

Similarly, underfeeding of normal mice has been shown to extend the life span. Weinruch, Walford, and their co-workers demonstrated that a 50% caloric restriction (alternate day feeding) initiated at weaning prolonged the life span of long-lived mice by 30%. Ca-

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loric restriction begun at 1 year of age prolonged lifespan of normal mice by 10–15%.[9–12]

Caloric restriction influences the life span of short-lived autoimmune mice even more dramatically. The life spans of the New Zealand Black/New Zealand White F_1 hybrid (B/W), BXSB, and MRL/l mice have been more than doubled by reducing caloric intake by 50%.[13–15] In B/W mice, underfeeding was associated with a decrease in circulating immune complexes (CICs) and decreased deposition of immunoglobulins in the glomerular capillaries. Mitogen-induced lymphocyte proliferation and generation of plaque-forming cells were better preserved later in life in undernourished animals as compared with age-matched animals fed *ad libitum*. In contrast to the beneficial effects of underfeeding, diets high in fat content have been shown to shorten the life span of female B/W mice.[16–20]

Three questions were addressed in our study. First, does undernutrition decrease the incidence and severity of vascular lesions in male MRL/l mice and increase the life span, as compared with animals fed *ad libitum*? Second, do diets which raise serum lipids increase the incidence and severity of vascular lesions? Third, do undernourished MRL/l mice exhibit the changes in immune function previously reported in undernourished B/W mice?

Materials and Methods

Mice

Weanling MRL/l and MRL/n male mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). The mice were housed 5 per plastic cage (18 × 29 cm) with wood chip bedding and *ad libitum* access to water and Formulab Chow (#5008, Ralston Purina Company, Richmond, Ind). Humidity (range 50–55%), temperature (range 70–75 C), and lighting (from 6:00 AM to 6:00 PM) remained constant for the duration of the experiment. Mice were housed in one room for the duration of the experiment, fed daily at 4:00 PM, and weighed once a week.

Nutritional Protocol

All mice were fed Formulab Chow for a 1-week period prior to being placed on special diets. A number of mice were killed at the end of the first week; they comprised a pre-diet, 1-month-old group. The remaining mice were divided among the six diets (Table 1) and maintained for either 8 weeks (to 3 months of age) or 16 weeks (to 5 months of age) before being killed. The effects of day-to-day variation on the immunologic assays were minimized by sacrificing equal numbers of mice from each diet group on any day that mice were killed.

Forty-five retired MRL/l breeders (9 males and 36 females) which had survived to an age of 5–6 months while being fed Formulab Chow (LC) *ad libitum* were individually housed and given either Diet II or Diet IV. Distribution of mice to the two diet groups was matched for age and sex. The mice were fed daily, weighed weekly, and followed until death, at which time the mice underwent autopsy, and the major organs were saved for histologic examination.

Diet Formulations

Diets I–V (Table 1) were purchased from ICN Nutritional Biochemicals (Cleveland, Ohio), and stored at 4 C. The ICN diets represent modifications of ICN semisynthetic diet #904663, a pelleted diet using vitamin-free casein (22.3% by weight) as a protein source, cornstarch (44.3%) as the major source of carbohydrates, and sucrose (11.2%), cellulose (11.0%), corn oil (5%), salt (mineral) mixture (6%), and vitamin mixture (0.2%) as the other major components. Mice fed Diet II received only 10 kcal/day; the diet contained twice the normal concentration of vitamins and minerals so that we could avoid inadvertent deficiencies. When the lipid or cholesterol content was increased (as in Diets III,

Table 1—Formulation of Experimental Diets

	Kcal/day*	% Fat	Type of fat	% Protein	% Cholesterol	% Cholic acid	Kcal/g
LC	18–20	6.5	Mixed veg.	25.3	0	0	3.3
I	18–20	5	Corn oil	22.3	0	0	3.6
II	10	5	Corn oil	22.3	0	0	3.3
III	18–20	20	Corn oil	22.3	0	0	4.3
IV	16–18	20	Corn oil	22.3	1	0.3	4.3
V†	16–18	20	18% coconut 2% corn oil	22.3	1	0.3	4.3

* Mice fed LC *ad libitum* consumed 18–20 kcal/day. Mice receiving Diets I, III, IV, and V were alloted 20 kcal/mouse/day; they consumed the amounts specified.

† The corn oil in Diet V (high calorie, high saturated fat) is to insure that the diet is not inadvertently deficient in essential fatty acids.

IV, and V) the cornstarch content was reduced accordingly. Uneaten food was discarded daily.

Statistics

Data were analyzed by one-way or two-way analysis of variance with the Tukey multiple-comparisons procedure.[21] Data are expressed as mean ± standard error (SE), and differences are considered significant if $P < 0.05$.

Analysis of Serum Samples

All mice were bled retroorbitally before being killed. Frozen sera were analyzed for total serum cholesterol, triglycerides, BUN, and total serum protein with the use of a Gilford Diagnostics System 202 (Gilford Instrument Laboratories, Cleveland, Ohio).[22-24]

Serum autoantibodies specific for double-stranded DNA (ds-DNA) were measured by a modification of a solid phase, enzyme-linked immunoassay technique.[25-27] For every 20 samples an assay control standard and a pooled normal mouse serum sample were also analyzed. MRL sera were scored as an optical density after we subtracted the amount of DNA binding activity in normal mouse serum.

Circulating immune complexes were detected with the use of a modification of the Raji cell assay.[29-30] Raji cells were fixed to the wells of a microtiter plate with glutaraldehyde. The wells were preincubated with human serum, washed, incubated with the test samples of murine serum, and again washed. Alkaline phosphatase-conjugated rabbit anti-mouse IgG (Miles Laboratories, Elkhart, Ind) was then added to each well, and the microtiter plates were incubated and then washed again. A reaction mixture of 0.2 ml of *p*-nitrophenyl phosphate (1 mg/ml) in 10% diethanolamine (pH 9.8) was added to each well. Plates were read with a Titertek Multiscan (Flow Laboratories, McLean, Va). Standard curves, using purified mouse IgG (Cappel Laboratories, West Chester, Pa) were included in each microtiter plate, and the absorbances of the test samples were converted to micrograms per milliliter of aggregated mouse IgG by extrapolation from the standard curves.

In Vitro PHA Proliferation and SRBC and TNP-PAA Plaque Induction

Spleens and cervical lymph nodes were aseptically removed to ice-cold Hanks' balanced salt solution (HBSS; Grand Island Biochemical Co., Grand Island, NY). Single-cell suspensions were derived from the tissues with glass, hand-held homogenizers (tissue grinder #7726, Corning Glass Works, NY). Suspensions were washed twice in HBSS, resuspended in RPMI-1640 (GIBCO) containing 5% heat-inactivated fetal calf serum (FCS), 1% L-glutamine, 100 IU of penicillin, and 100 μg/ml streptomycin (complete RPMI), and counted on a hemacytometer.

Spleen and lymph node cultures (0.2 ml/well) were cultured in complete RPMI-1640 in flat-bottomed microwells (Costar, Cambridge, Mass) at a concentration of 1×10^6 cells/ml. Phytohemagglutinin (Purified PHA, Wellcome Reagents Limited, Beckenham, England) was added to a final concentration of 1.0 μg/ml, a concentration found to elicit optimal responses from MRL lymphocytes. One lot of PHA and one lot of FCS were used for the entire experiment. Microwells were pulsed for 8 hours with 1.0 μCi of ^{3}H-thymidine on the third day of culture. Data are expressed as counts per minute.

In vitro plaque-forming cell (PFC) cultures were set up as previously described.[31] Briefly, splenocytes were cultured in plastic Petri dishes with either sheep red blood cells (SRBC) or trinitrophenylated polyacrylamide beads (TNP-PAA) for 4 days. The cells were then assayed for number of anti-SRBC or anti-TNP plaque-forming cells. Data are reported as direct or reverse PFCs per 10^6 viable cells.

Histology

An 18-gauge needle was introduced into the left ventricle of the heart, and each mouse was perfused with 10 ml of 10% buffered formalin containing 2% zinc sulfate. The torso from above the heart to below the kidneys was transferred to and kept in formalin for at least 1 week. The heart, the aorta and its branches, both lungs, the liver, and one kidney were embedded in paraffin, sectioned, and stained with periodic acid-Schiff stain (PAS). Histologic observations were made without knowledge of the age, substrain, or diet grouping of the mouse being examined.

Mice from the retired breeder study were autopsied within 24 hours of death. The torso sections were transferred to 10% formalin and treated as above.

Results

Weight and Weight Gain

MRL/l and MRL/n on arrival had an average weight of 18.7 and 19.3 g, respectively. Figure 1 illustrates the gain in weight of MRL/l mice fed various diets for 16 weeks. The 10 kcal/day group (Diet II) showed a slight but consistent weight gain over the duration of the feeding regimen. Mice receiving the other five diets demonstrated rapid early weight gain followed by a relative

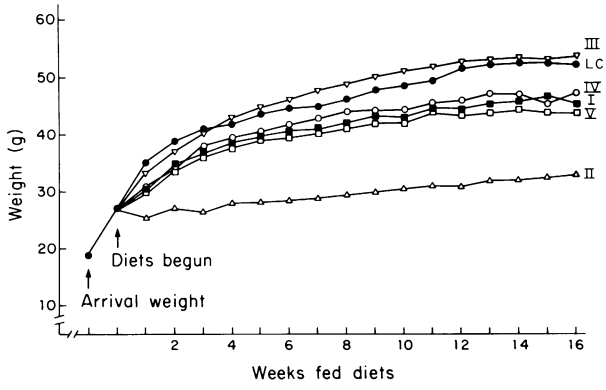

Figure 1 – Changes in weight of MRL/l mice fed various diets. Weanling, male MRL/l mice were fed LC *ad-libitum* for 1 week and then randomly allocated to the 6 diets for 16 weeks before sacrifice. Data represent the mean weekly weights of 35 mice fed each diet.

plateau. After 16 weeks, mice receiving a diet containing 20% corn oil (Diet III) were 17% heavier ($P < 0.05$) than mice receiving a similar diet with 5% corn oil (Diet I). Mice receiving the cholesterol-containing, high-fat diets (Diets IV and V) ate less than the other mice fed *ad libitum*; after 16 weeks they weighed 15% less than mice receiving the high-fat diet without cholesterol (Table 2).

MRL/n mice fed the calorically restricted diet (Diet II) had a growth curve and adult body weight identical to the MRL/l mice of Figure 1. MRL/n mice receiving the *ad libitum* diets tended to be slightly heavier than the diet-matched MRL/l mice. After the mice were on the diets 16 weeks, the differences between strains averaged 10% (Table 2).

Thirteen percent of the MLR/l mice died during the 16 weeks of test diet feeding, with 70% of the deaths occurring in the last 2 weeks. Differences in deaths among diet groups ranged from 5% (LC) to 16% (III), but were not significant because of the small number of total deaths (28 of 210 mice).

Effects of Late Diet Intervention on Survival

Retired MRL/l breeders were switched from *ad libitum* LC to either Diet II or Diet IV at an age of 5–6 months. At the initiation of the diet change the retired breeders had an average weight of 42 ± 4 g (mean ± SD). Mice switched to Diet II lost weight for three weeks before reaching a stable weight of 32 ± 2 g. By the fourth to sixth week their appearance was noticably improved: lymph nodes shrank, the scarring and sores across the upper back healed, and the mice were as physically active as 2-month old mice. These mice remained at their plateau weight until they died.

Mice switched to the high-fat, cholesterol-containing diet (Diet IV) continued to maintain a stable weight of 42 ± 4 g, but they underwent a precipitous weight loss before death. Two weeks and 1 week before death the average weights were 36 and 33 g, respectively. Their physical appearance continued to deteriorate during the experiment: massively enlarged lymph nodes, necrosis of the tips of the ears, and physical inactivity were the norm. One-third of these mice developed extremely severe scarring, open sores, and hair loss across the upper back. Self-grooming seemed to contribute to this condition.

The underfed and high-fat–fed retired breeders had comparable mortality rates for the first 6 weeks after diet initiation (Figure 2). After the sixth week the death rate among calorically restricted mice was dramatically slowed. Thus, of the 12 undernourished mice alive at 6 weeks, 8 were still alive at 35 weeks and 5 were still alive at 54 weeks. In contrast, of the 13 mice fed *ad libitum* alive at 6 weeks, all but 3 were dead by 11 weeks, and none survived beyond 20 weeks.

Spleen and Lymph Node Cell Yields

For the 5-month-old MRL/l mice fed *ad libitum* the spleen and lymph nodes taken together made up approximately 10% of total body weight, with many lymph nodes exceeding the kidneys in size. The number of cells obtained from the spleens of MRL/l mice averaged two to four times the number obtained from MRL/n mice (Table 2). For the MRL/l substrain, caloric restriction (Diet II) resulted in a significantly lower absolute and relative (to body weight) splenic cell yield. For the MRL/n substrain, caloric restriction resulted

Table 2 – Effect of Diet on Splenic Cell Yields*

	LC	I	II	III	IV	V
MRL/l Spleen cells (10^{-6})	274 ± 30^{a}	245 ± 21^{a}	115 ± 11^{b}	290 ± 22^{a}	257 ± 33^{a}	242 ± 27^{a}
Body weight (g)	51 ± 1^{a}	45 ± 1^{b}	32 ± 1^{c}	53 ± 2^{a}	46 ± 1^{b}	44 ± 1^{b}
Yield/100 g body wt	537^{a}	544^{a}	359^{b}	547^{a}	559^{a}	550^{a}
MRL/n Spleen cells (10^{-6})	89 ± 5^{a}	61 ± 3^{b,c}	53 ± 4^{b}	71 ± 5^{c}	65 ± 4^{c}	69 ± 5^{c}
Body weight (g)	54 ± 1^{a}	52 ± 1^{a}	31 ± 1^{b}	62 ± 2^{c}	49 ± 2^{a}	49 ± 1^{a}
Yield/100 g body wt	165^{a}	117^{b}	171^{a}	115^{b}	131^{a,b}	141^{a,b}

* Five month old mice had been fed diets for 16 weeks. Data are expressed as mean ± SEM (n = 15 mice/diet).
^{a,b,c} For each line, values not sharing a common superscript are significantly different at $P < 0.05$ (ANOVA).

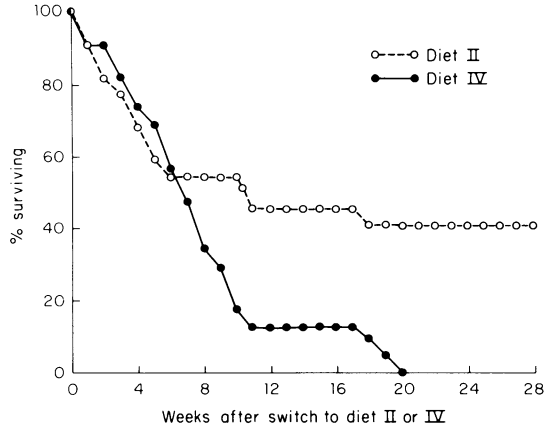

Figure 2—Effect of late-life dietary intervention on longevity. At an age of 5-6 months retired MRL/l breeders were switched from LC to either Diet II or Diet IV.

in a lower absolute number of splenic cells, but not a lower relative yield (Table 2). Cervical lymph nodes from MRL/l mice undergoing caloric restriction (Diet II) were far smaller than nodes from mice receiving other diets, both in absolute terms and in relation to spleen or body weight.

At 3 months of age splenic cell yields of MRL/l mice were one to two times the MRL/n yields, reflecting splenic hypertrophy in the MRL/l mice. For both substrains caloric restriction (Diet II) resulted in significantly lower absolute cell yields, but no change in cell yield relative to body weight (data not shown).

Serum Cholesterol, Triglycerides, BUN, and Total Serum Protein

At 1 month of age the two MRL substrains had similar serum cholesterol, serum triglycerides, BUN, or total serum protein: MRL/l and MRL/n mice had serum cholesterol concentrations of 208 ± 6 and 196 ± 6 mg/dl, triglyceride concentrations of 187 ± 15 and 186 ± 21 mg/dl, BUN values of 22 ± 2 and 22 ± 2 mg/dl, and total serum protein levels of 6.0 ± 0.5 and 5.9 ± 0.5 g/dl, respectively.

At 5 months of age MRL/n mice had higher serum cholesterol levels than MRL/l mice for all diets (Table 3). The difference was significant for Diets I, III, and V ($P < 0.05$). For both substrains the high-fat diets resulted in relatively higher serum cholesterol concentrations, with the diet containing saturated fat and cholesterol (Diet V) resulting in the highest values.

Five-month-old MRL/n mice fed diets LC and I also had significantly higher serum triglyceride levels than MRL/l mice (Table 3). For both substrains the high-fat diets (III and V) tended to result in relatively lower serum triglyceride levels, with the diet containing high polyunsaturated fat and cholesterol (Diet IV) resulting in the lowest values.

Five-month-old MRL/n mice fed LC had lower BUN values than MRL/l mice ($P < 0.05$). For both substrains the high-fat diets resulted in lower BUN values (Table 3) than the low-fat diets. MRL/n mice also had lower total serum protein concentrations than MRL/l mice; the averages for all diets were 7.9 ± 0.1 and 8.5 ± 0.1 g/dl, respectively ($P < 0.05$). There were no significant differences due to diet for total serum protein (Table 3). The observation that calorically restricted mice (Diet II) did not have lower serum protein levels than the mice fed *ad libitum* suggests that although they were receiving less total dietary protein, they should not be considered protein-malnourished.

PHA-Induced Proliferation

Spleen cells (but not lymph node cells) from 3-month-old MRL/l mice fed high-fat diets showed significantly

Table 3—Effects of Diet on Serum Cholesterol, Triglycerides, and BUN*

Strain	LC	I	II	III	IV	V
Serum total cholesterol (mg/dl)						
MRL/l	187 ± 13[a]	243 ± 15[a]	212 ± 14[a]	242 ± 16[a]	315 ± 27[b]	358 ± 26[b]
MRL/n	218 ± 8[a]	314 ± 8[b]	246 ± 6[a]	323 ± 7[b]	320 ± 8[b]	436 ± 20[c]
Serum triglycerides (mg/dl)						
MRL/l	155 ± 19[a]	164 ± 14[a]	151 ± 18[a]	144 ± 16[a]	120 ± 15[a]	123 ± 12[a]
MRL/n	226 ± 22[a]	269 ± 17[a]	137 ± 7[b]	187 ± 10[c]	124 ± 5[b]	167 ± 8[c]
Blood urea nitrogen (mg/dl)						
MRL/l	37 ± 3[a]	29 ± 2[b]	28 ± 2[b]	25 ± 3[b,c]	21 ± 1[c]	24 ± 1[b,c]
MRL/n	29 ± 2[a]	30 ± 2[a]	24 ± 1[b]	20 ± 1[b]	21 ± 1[b]	21 ± 1[b]
Serum total protein (g/dl)						
MRL/l	9.0 ± 0.3[a]	8.6 ± 0.2[a]	8.6 ± 0.2[a]	8.2 ± 0.2[a]	8.5 ± 0.1[a]	8.7 ± 0.3[a]
MRL/n	7.8 ± 0.1[a]	7.8 ± 0.1[a]	7.6 ± 0.1[a]	7.6 ± 0.1[a]	8.0 ± 0.1[a]	8.0 ± 0.1[a]

* Five-month-old mice had been fed diets for 16 weeks. Data expressed as mean ± SEM (n = 10 mice/diet).
[a,b,c.] For each line, values not sharing a common superscript are significantly different at $P < 0.05$ (ANOVA).

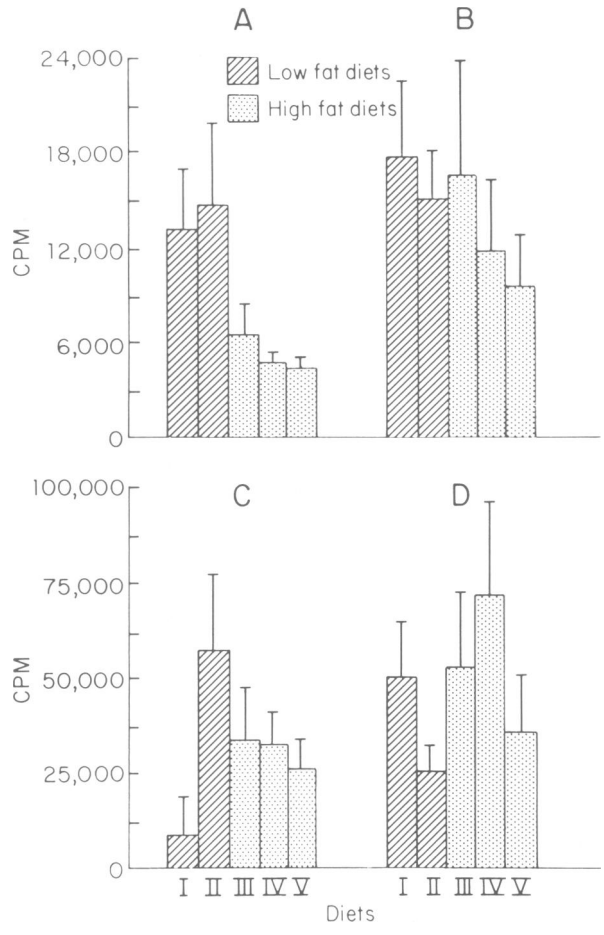

Figure 3—PHA proliferation. Spleen and lymph node cells obtained from MRL/l and MRL/n mice fed diets for 8 weeks were incubated with PHA for 3 days in microwell cultures. Data are reported as counts per minute (cpm) (mean ± SEM; n = 14 mice/diet). **A**—MRL/l spleen cells. **B**—MRL/l lymph node cells. **C**—MRL/n spleen cells. **D**—MRL/n lymph node cells. Notice the difference in scale between substrains.

lower proliferative responses to PHA than spleen cells from mice fed low-fat diets (Figure 3). MRL/n proliferative responses were on the average three to four times greater than MRL/l responses. No dietary effect on spleen or nodal cell responses to PHA could be shown for MRL/n mice. Lymph node and spleen cells cultured in the absence of PHA incorporated 700 and 1000 cpm, respectively, with no significant differences between substrains or among diets (data not shown).

Cultures of spleen cells from 5-month-old MRL/l mice had 60% lower proliferative response to PHA than cultures from 3-month-old mice. Dietary effects on PHA responses were similar to those observed with cultures from 3-month-old MRL/l mice. Cultures of MRL/l lymph node cells from the older mice showed 85% lower proliferative responses than those of the younger mice. At 5 months of age the PHA proliferative response of lymph node cells from MRL/l mice on high-fat diets was significantly lower than the response of the cells from MRL/l mice on lower fat diets, although there had been no significant diet effect at 3 months. In contrast to the MRL/l results, the proliferative responses of MRL/n spleen and lymph node cells were 40% higher for the 5-month-old than for the 3-month-old mice; and as had been true at 3 months of age, there was no significant difference associated with dietary fat content (data not shown).

Direct and Reverse PFC

For both MRL/l and MRL/n 16 weeks of caloric restriction (Diet II) resulted in higher direct TNP-PFCs (Figure 4). MRL/n PFCs were an average of four times higher than diet-matched MRL/l PFCs. The difference between substrains was significant for all diets. Reverse PFC, a measurement of total B-cell activation, demonstrated similar relationships: for the MRL/l substrain

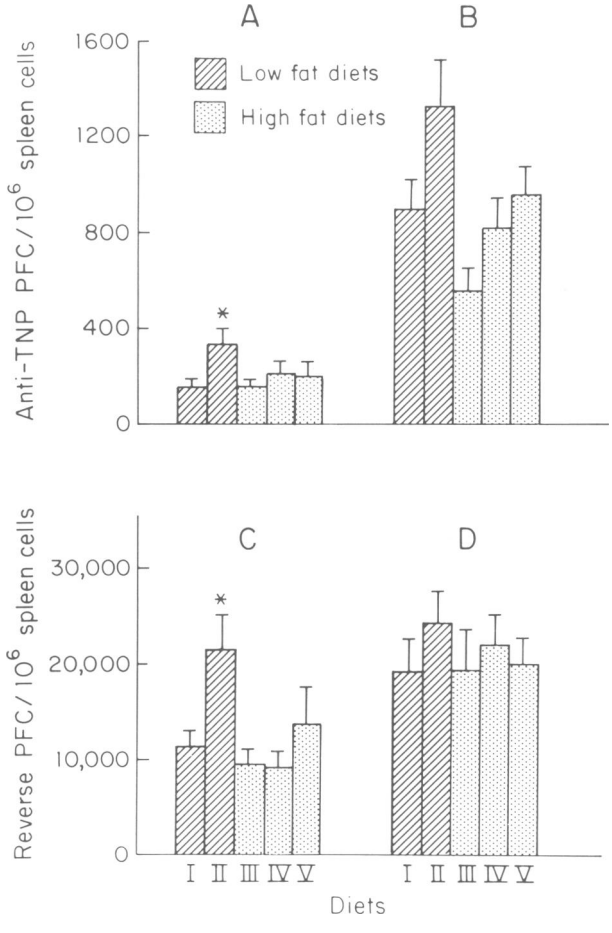

Figure 4—*In vitro* plaque induction. Splenocytes obtained from MRL/l and MRL/n mice fed the various test diets for 16 weeks were cultured for 4 days with TNP-PAA. Data are reported as PFCs/10^6 viable cells (mean ± SEM; n = 13 mice/diet). **A**—MRL/l direct PFC. **B**—MRL/n direct PFCs. **C**—MRL/l reverse PFCs. **D**—MRL/n reverse PFCs.

(but not for MRL/n) caloric restriction resulted in a 100% increase in PFCs. Between substrains, MRL/n responses averaged twice the MRL/l responses. The difference between substrains was significant for all diets except Diet II. Direct and reverse PFCs were also generated by *in vitro* culture of spleen cells with sheep red blood cells. There were no significant diet effects (data not shown).

Anti-DNA Antibodies

At 5 months of age calorically restricted MRL/l mice had an anti-DNA value of 0.43 ± 0.04. While this was 20% lower than the average antibody concentration of the other diets, which ranged from 0.52 ± 0.06 to 0.55 ± 0.05, the difference did not reach statistical significance. For every diet the anti-DNA antibody concentrations in the MRL/n mice were 20–40% lower than those of the MRL/l mice, and these differences were statistically significant ($P < 0.05$).

Circulating Immune Complexes

Three-month-old MRL/l mice had CIC levels ranging from 410 ± 30 to 520 ± 30 μg/ml. There were no significant differences among diets. At 5 months of age calorically restricted mice had CIC levels of 590 ± 40 μg/ml. This mean was 10% lower than the average of the other diets, which ranged from 620 ± 30 to 690 ± 60 μg/ml. None of the differences among diets were statistically significant, but all values were significantly higher than those of 3-month-old mice fed the same diets ($P < 0.05$). All MRL/n mice had CIC levels below 300 μg/ml, with no significant differences associated with either diet or age.

Histopathologic Features of Mice Killed at 5 Months of Age

Mice of both substrains fed Diet IV or Diet V (the two cholesterol-containing diets) had extremely fatty

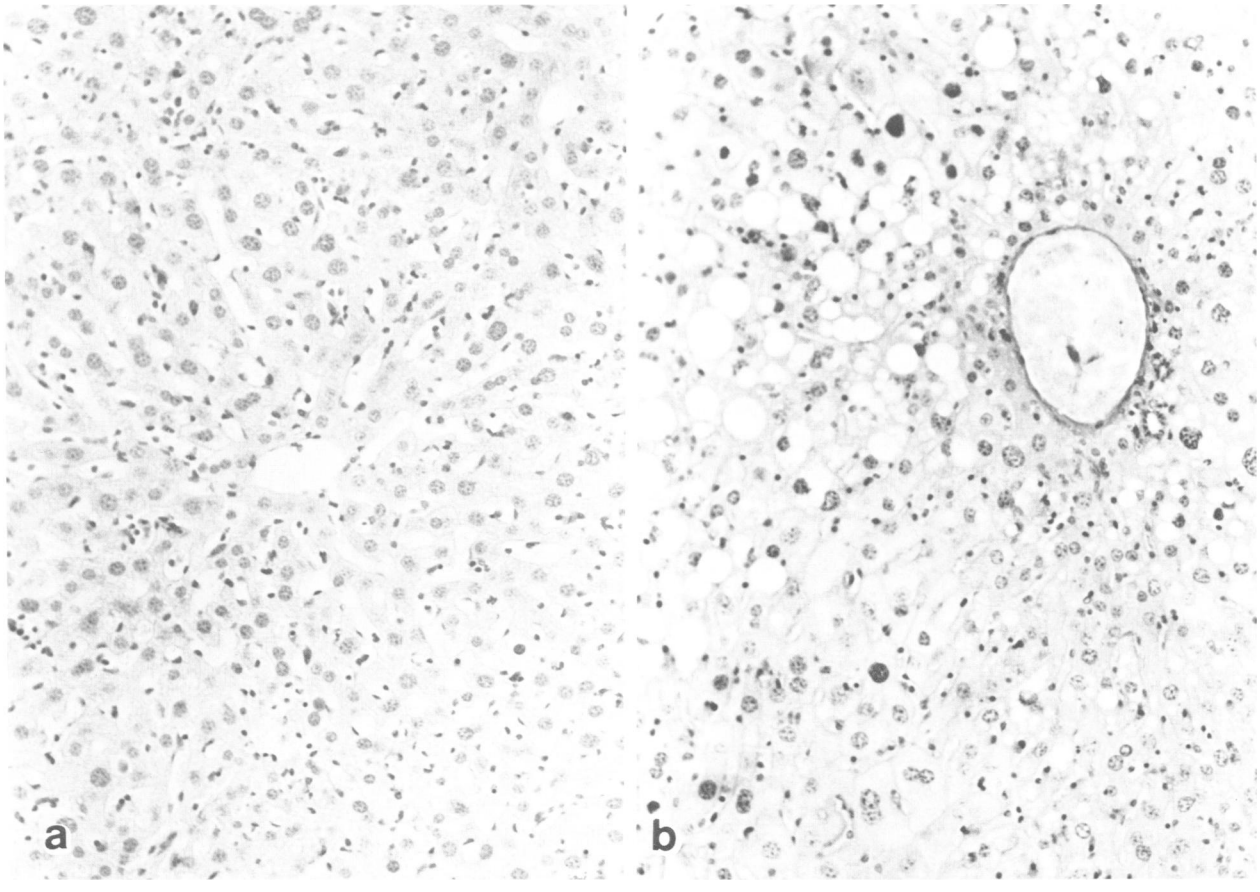

Figure 5 — Appearance of the liver. **a** — Liver of a 5-month-old MRL/l mouse fed LC. Hepatocytes lack visible cytoplasmic vacuoles and have uniform nuclei. Scattered lymphocytes are seen throughout the lobule. **b** — Liver of a 5-month-old mouse fed Diet IV (high PUFA with cholesterol), showing many hepatocytes distended by cytoplasmic lipid vacuoles. Notice the nuclear pleomorphism of hepatocytes. (PAS, × 220)

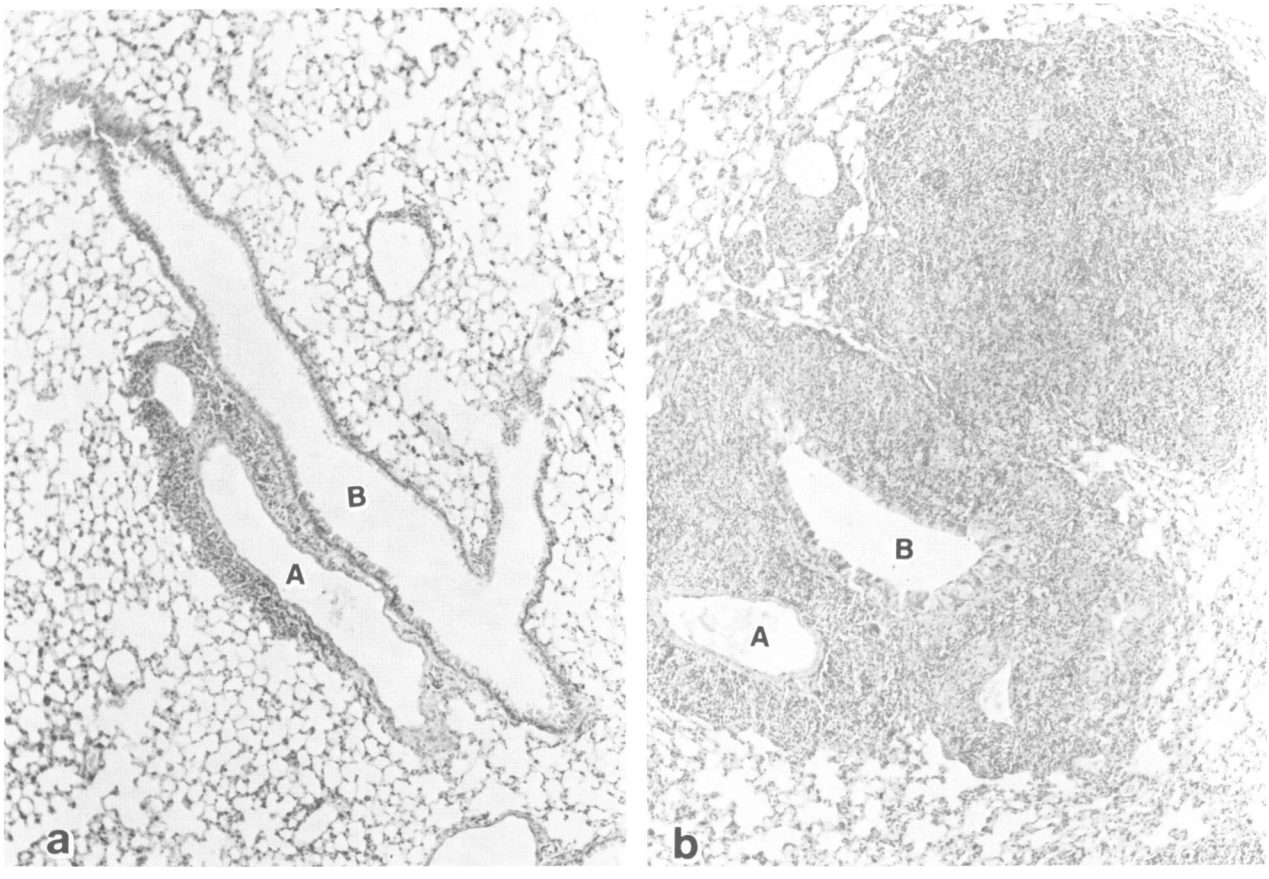

Figure 6—Example of pulmonary lymphoproliferation in MRL/l mice. **a**—A 1+ lesion showing narrow, periarterial, lymphoid infiltrate. **b**—A 4+ lesion characterized by massive, confluent, periarterial, peribronchial, and parenchymal lymphoid infiltrate. *A*, artery; *B*, bronchus. (PAS, ×112)

livers. Animals fed the other diets (including Diet III, a high-fat diet devoid of cholesterol) did not have accumulations of fat in their livers. Fatty change (Figure 5) was characterized by either a single, clear vacuole which distended the hepatocyte and displaced its nucleus (Diet IV), or by multiple, smaller vacuoles (Diet V). Frequently, the fatty change was associated with considerable variation in nuclear size and hyperchromatism. This nuclear pleomorphism was not observed in nonfatty livers.

The severity of the lymphoproliferation could be best assessed histologically in the lung, because many of the enlarged lymph nodes and the spleen had been utilized for the immunologic studies. Lymphocytes and macrophages typically infiltrated perivascular and peribronchial interstitial tissues of both lungs in a diffuse fashion (Figure 6). Lesions were graded on a scale of 0 to 4+, the latter indicating confluent, extensive lymphoproliferation. Table 4 shows that all mice of the MRL/l substrain showed considerable lymphoproliferation with no significant differences related to diet. Mice of the MRL/n substrain were virtually free of lymphoproliferation at 5 months of age.

The typical glomerular lesions known to occur in

Table 4—Effects of Diet on Lymphoproliferation, Arterial Lesions, and Glomerulonephritis*

	LC	I	II	III	IV	V
Lymphoproliferation†	2.2 ± 0.3[a]	2.1 ± 0.2[a]	1.9 ± 0.2[a]	2.3 ± 0.2[a]	2.5 ± 0.3[a]	2.2 ± 0.3[a]
% With aortic lesions	22	54	54	15	17	23
% With renal lesions	67	44	60	50	55	58
Glomerulonephritis†	2.1 ± 0.3[a]	1.8 ± 0.2[a]	1.0 ± 0.3[b]	1.7 ± 0.2[a]	1.5 ± 0.2[a]	1.4 ± 0.2[a]

* Five month old MRL/l mice had been fed diets for 16 weeks (MRL/n results are presented in the text). Data are expressed as the mean ± SEM (n = 9–13 mice/diet).

† Lymphoproliferative invasion of the lungs ranged from 0 (lowest) to 4 (highest). Glomerulonephritis was scored in a similar fashion.

[a,b] For each line, values not sharing a common superscript are significantly different ($P < 0.05$).

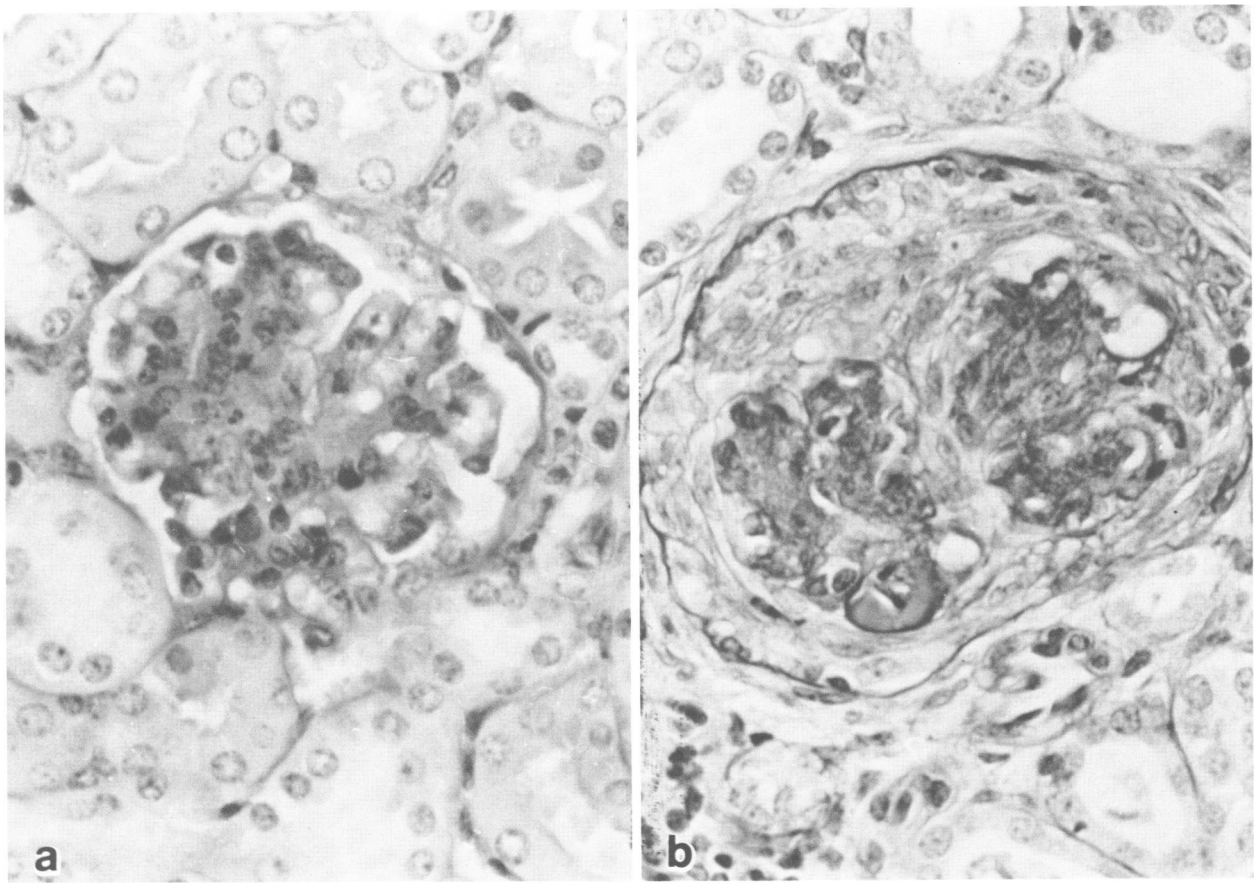

Figure 7 — Glomeruli from two MRL/l mice illustrating the changes seen at 5 months of age. **a** — Moderate (2+) glomerulonephritis characterized by focal hypercellularity. **b** — Advanced (4+) lesion showing total obliteration of the urinary space by a crescent, and sclerosis and "wire-loop" changes of the glomerular tuft. (PAS, ×550)

autoimmune-prone mice were observed in the MRL/l mice. Lesions were graded on a scale of 0 to 4+, the latter indicating severe sclerosis and obliteration of a majority of glomeruli. Intermediate grades were assigned to various stages of proliferative glomerulonephritis (Figure 7). A dietary effect for glomerulonephritis was evident: caloric restriction minimized severity of the damage, while among the *ad libitum* diets there was little difference regardless of the percentage or nature of the fat content or the presence or absence of cholesterol (Table 4). Mice of the MRL/n substrain were virtually free of glomerular lesions at 5 months of age.

Arterial lesions were found in the aorta, in its major muscular branches, and in arteries within the kidney. The vast majority of lesions were typical examples of active necrotizing arteritis with fibrinoid necrosis (Figure 8a). Occasionally, a fibromuscular intimal plaque was seen. As can be seen in Table 4, the incidence of lesions of the renal arteries was not affected by changes in lipid, cholesterol, or calorie content. Aorta and aortic branch lesions were more frequent in the groups fed the casein-based, low-fat diets (I and II). Lipid-containing lesions were rarely (<5%) observed at 5 months of age.

The heart was examined for the presence of lesions in the myocardium and coronary arteries. Occasional hearts showed a few foci of slight interstitial fibrosis of the myocardium with no particular pattern of distribution. No myocardial infarcts were seen. The coronary arteries were unremarkable.

Histopathology of Retired Breeders

Retired breeders had been fed either Diet II (restricted calories) or Diet IV (high-fat with cholesterol) from 5–6 months of age until death. Mice fed Diet IV did not have the extremely fatty livers at the time of death that similarly fed mice showed when killed at 5 months; only 30% showed mild to moderate visible lipid deposition. All of the mice displayed some degree of nuclear pleomorphism of hepatocytes. The average severity of this change in the undernourished mice was half that found in the fat-fed mice ($P < 0.05$).

Necrotizing arteritis involving the intrarenal branches

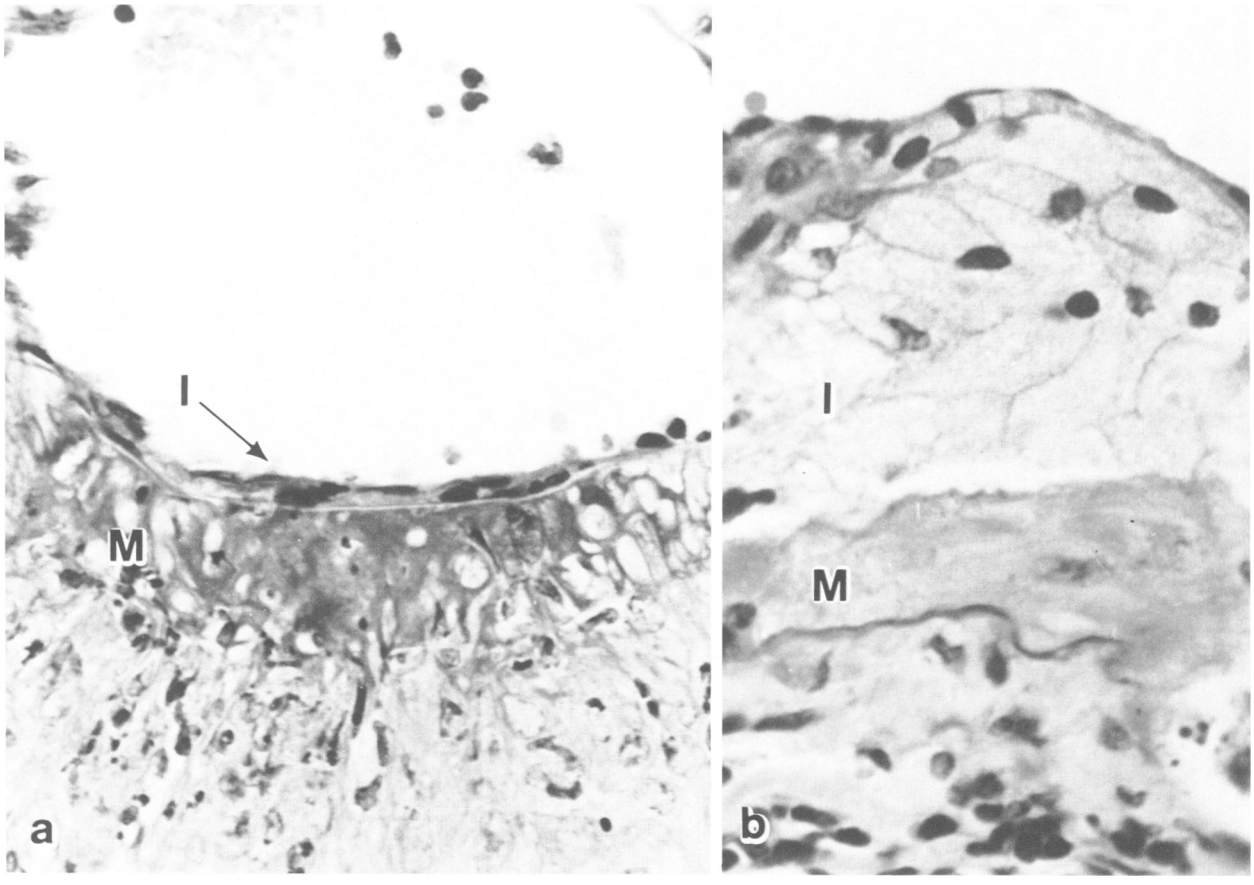

Figure 8a – Necrotizing arteritis of intrarenal artery of an MRL/l mouse killed at 5 months of age. Notice the fibrinoid necrosis of the media and inflammatory infiltrate and debris in the adventitia. No fat is apparent, and the intima is minimally thickened. *I*, intima; *M*, media. (PAS, ×970) **b** – Atheromatous lesion of intrarenal artery of retired MRL/l breeder that died at 8.5 months of age and had been fed Diet IV for the last 3 months of its life. The intima is greatly thickened by numerous foam cells. (H&E, ×1320)

of the renal artery was evident in 63% and 73% of the mice fed Diet II and Diet IV, respectively. The difference in incidence was not significant; nor were the differences in incidence of aortic lesions (22% and 23%, respectively) or aortic branch lesions (67% and 69%, respectively). However, 43% of the mice fed Diet IV had lipid-containing lesions, characterized by the presence of intimal foam cells (Figure 8b) and occasionally cholesterol crystals located deep within a thickened intima and covered by a fibrous cap. An example of a typical atheroma is shown in Figure 9a. Such atheromatous lesions were not seen in the underfed (Diet II) retired breeders. In addition, aneurysmal dilatations of aortic branches were observed in two of the fat-fed mice. Figure 9b illustrates one of these lesions: it is characterized by a sharp interruption of the arterial wall and dilatation and thickening of a portion of the vessel circumference. Focal calcification and probably extracellular lipid were visible within the thickened wall of the aneurysm.

When pathologic examinations were conducted on mice that had been allowed to die of natural causes, the MRL/l mice demonstrated much more serious damage to the heart than had been evident in animals killed at 5 months of age. Focal calcium deposition was found in the myocardium of 44% and 38% of the underfed and the high-fat–fed mice, respectively (Figure 10). Both groups showed some degree of interstitial scarring of the myocardium, but the average severity was more than twice as high in the mice fed the high-fat, cholesterol-containing diet.

Discussion

There have been a number of investigations on the effects of modifications of caloric intake, lipid content, lipid type, and cholesterol content on life span. Weaning-initiated severe (50%) caloric restriction had repeatedly been shown to greatly extend the life span of NZB, B/W, and MRL/lpr mice, all autoimmune-prone strains.[13–15] In contrast, high fat diets have been shown to accelerate the development of autoimmune

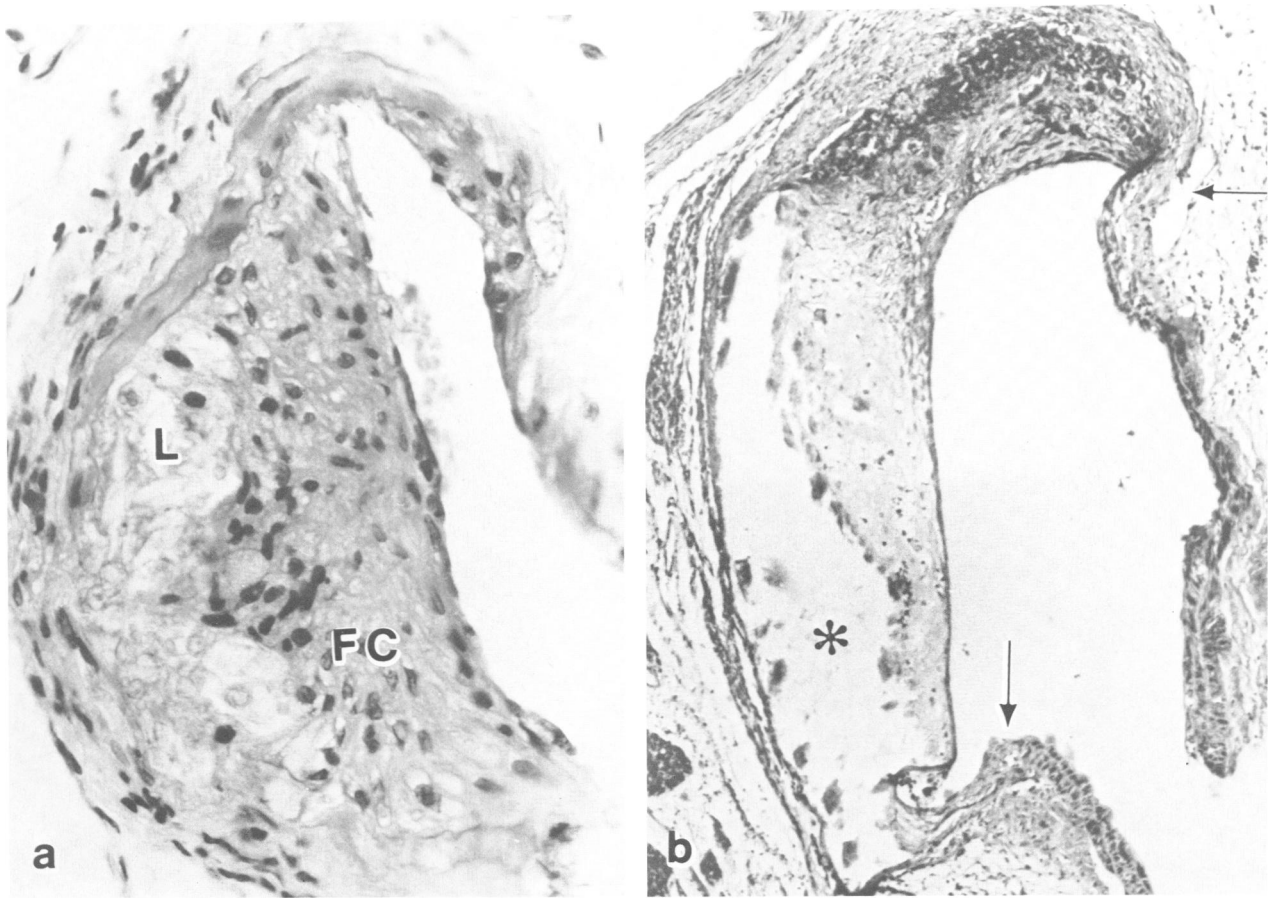

Figure 9 – Representative arterial lesions in retired MRL/l breeders fed Diet IV from 5 months of age until death. **a** – Retroperitoneal branch of the abdominal aorta showing a typical atheromatous plaque. It is characterized by eccentric intimal thickening, accumulation of lipid (*L*), including cholesterol crystals deep within the thickened intima, and an overlying fibrous cap (*FC*). (PAS, ×265) **b** – Section of carotid artery with aneurysmal dilatation (*between arrows*). The media shows abrupt attenuation. The wall of the aneurysm is greatly thickened and focally calcified (*). Most of the calcified area appears empty because of loss of hard calcified material during sectioning. (Masson's trichrome, ×160)

disease and shorten the life span of female B/W mice.[16-20,32] Relationships between high-fat diets and an increase in the incidence and severity of glomerulonephritis and arteriosclerosis have also been demonstrated.[20,32] In the present study we fed male MRL/l and MRL/n mice a number of high-fat and low-fat diets with and without added cholesterol in an attempt to clarify the effects of diet on immunocompetence, autoimmune disease, and histopathologic sequelae.

Caloric restriction resulted in minimal weight gain over the 16-week duration of the present study. Mice fed *ad libitum* approximately doubled their initial body weights over the same time interval. With the exception of the calorically restricted group, the MRL/n mice were always heavier than diet-matched MRL/l mice in spite of the fact that by 5 months of age the latter had the massively enlarged lymph nodes typical of the uncontrolled lymphoproliferative condition. This suggests that the MRL/n mice were able to maintain either a larger lean body mass or a higher fat deposition rate than were their congenic, autoimmune peers. It is of interest to note that the inclusion of cholesterol and cholic acid in two of the high-fat diets (IV and V) resulted in lower body weights than those observed in mice fed Diet III; measurement of food disappearance from the cages has shown that the differences in body weight were proportional to the differences in food consumption (unpublished observations).

Late-life initiation of caloric restriction resulted in a dramatic difference in survival. MRL/l mice switched from LC to a calorically restricted diet showed no difference in survival for the first 6 weeks. Mice that survived this transition period had greatly extended life spans. The appearance of health, activity, and the maintenance of body weight up to the point of death were in sharp contrast to the progressive physical deterioration of the animals fed high-fat diets. The relative extension of the life span observed exceeds those previously achieved

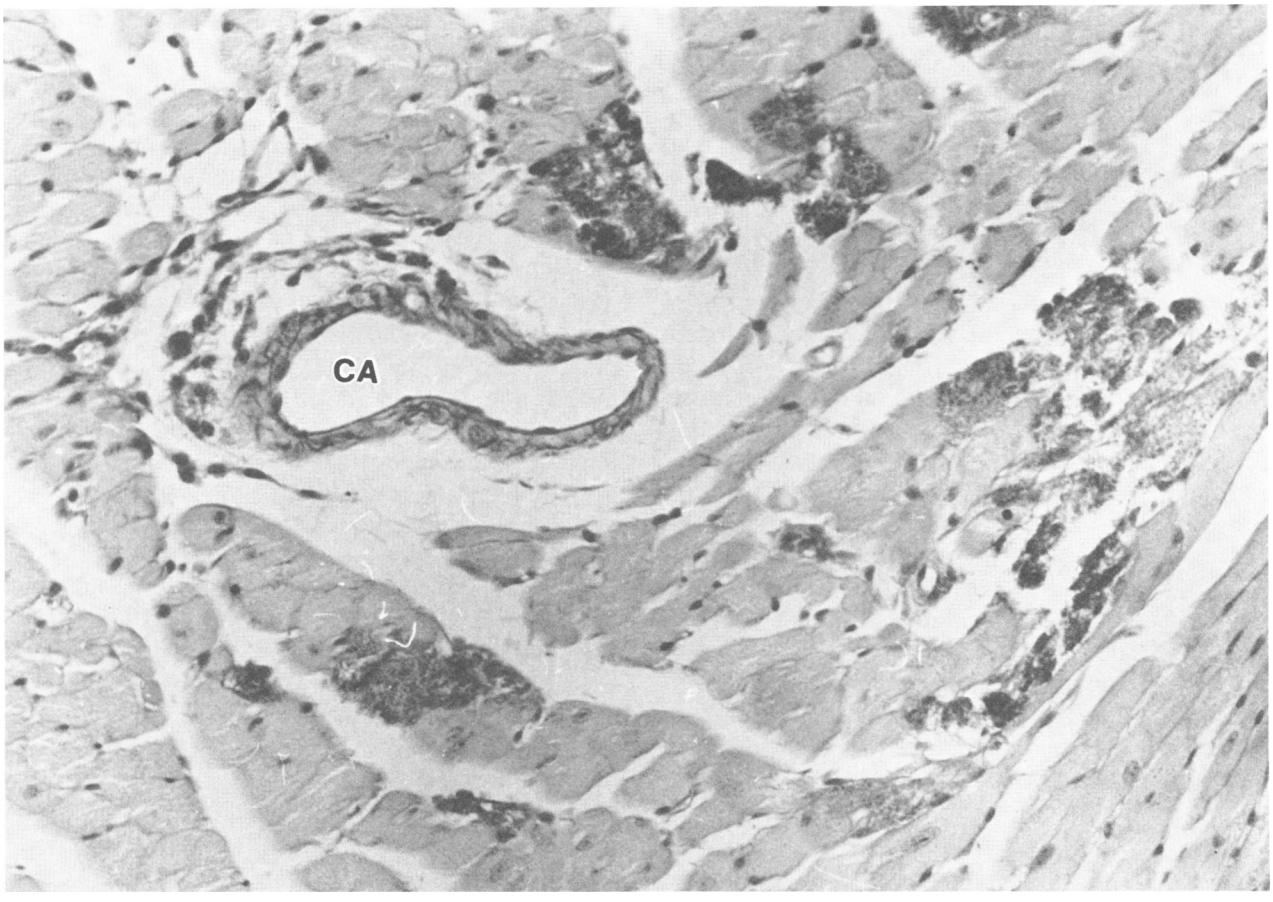

Figure 10 — Myocardium of retired breeders showing calcified scars replacing myofibers. A coronary artery branch (*CA*) is unremarkable. (PAS, ×300)

through late-life initiation of a low-calorie diet either in long-lived mice[11] or other strains of autoimmune mice.[15]

Young MRL mice fed LC had higher serum cholesterol levels than have been reported for similarly fed mice of other strains.[19,33] Approximately 75% of the genetic lineage of the MRL strain is derived from LG/J mice.[2] We found that LG/J mice also have initial cholesterol levels greater than 150 mg/dl and post-diet levels over 300 mg/dl (unpublished observations). Thus, MRL/l and MRL/n mice can be characterized as having a genetic predisposition to high serum cholesterol levels and a susceptibility to hypercholesterolemia when fed diets containing exogenous cholesterol. The use of casein as a protein source in the semisynthetic diets probably also contributed to the hypercholesterolemia.[34-36]

The gross appearance of the lymphoid tissues and the number of spleen cells recovered from 5-month-old MRL/l mice demonstrated that caloric restriction limits the lymphoproliferation characteristic of this strain. A reduction in caloric intake also resulted in the lowest levels of anti-DNA antibodies, CICs, and glomerulonephritis. These results are in accord with previously published reports on the effects of caloric restriction on the progression of autoimmune disease.[13-20]

At the time the MRL mice were killed high-fat diets had not led to significantly higher CIC levels or more severe glomerulonephritis than were found in mice fed the low-fat diets. This is in contrast to the reported accelerating effect of high-fat diets on disease progression in female B/W mice.[16-20,32] It is possible that MRL/l mice followed to obvious morbidity would have had a more severe autoimmune disease and clearer effects of the high-fat diets.

MRL/l mice manifested lower *in vitro* spleen cell responses to the T-cell mitogen PHA and the T-cell–independent B-cell antigen TNP-PAA than did cells from MRL/n mice. The differences were evident at 3 months and greater at 5 months of age. As the MRL/l mice are known to undergo a massive and progressive proliferation of Thy 1.2+, Lyt1+ T cells in all lymphoid tissues the dilution of competent cells with these nonresponsive T cells is the most likely cause of the differ-

ence in immunocompetence between the congenic substrains.[1-3]

The PFC response to the T-independent antigen TNP-PAA was enhanced by caloric restriction in both substrains. This observation is consistent with the view that protein-energy malnutrition may reduce specific and nonspecific suppressor activity.

Within the MRL/l substrain, mice fed high-fat diets had lower splenic PHA responses than mice fed low-fat diets. PFC generation did not show any relation to fat intake. High fat intake, either polyunsaturated (PUFA) or saturated, has previously been reported to suppress the T-cell response to mitogens, increase thymolytic activity, and prolong skin graft survival, but not to affect B-cell functions as assessed by the LPS mitogenic response, IgG concentrations, PFCs, or delayed hypersensitivity responses.[32,37-41] Locniskar et al also noted that lymph nodes were less likely to show the immunosuppressive effect of PUFA on T-cell mitogen responses than were spleen cells.[37]

Three mechanisms potentially link plasma lipid status to T-cell immune function. Low density and very low density lipoproteins inhibit mitogenic proliferation.[42] Increases in plasma cholesterol result in increases in the lymphocyte membrane cholesterol/phospholipid ratio, which in turn result in increased viscosity and decreased responsiveness to mitogens.[43,44] Lastly, it is possible that lipid and cholesterol levels modulate the immune system through effects on prostaglandin E_2 synthesis.[45] Investigations of these mechanisms are in progress, and it is of interest to note that the accompanying paper demonstrates strong correlations between cholesterol levels and prostaglandin synthesis.

Protein restriction has been reported as having beneficial immunomodulatory and life span extending effects similar to those reported for caloric restriction, but the effects are not as great, and they seem to require a dietary protein content of 6% or less.[13-15,46-48] Such low levels of protein intake in an isocaloric diet result in lower serum total protein and serum albumin levels. In our study, Diet II, although providing less total protein than Diet I, did not result in lower serum protein levels, suggesting that results attributed to caloric restriction were not due to the concomitant protein restriction.

The effects of five different diet regimens on male and female B/W vascular disease have previously been reported.[20] At an age when approximately 50% of the mice had died, those still alive were found to have a low incidence of vascular lesions if they had been fed LC or were calorically restricted. Exclusive of the restriction diet, the other semisynthetic diets resulted in a high incidence of fatty proliferative vascular lesions regardless of whether they were high (20%) or low (5%) fat or contained saturated or polyunsaturated fatty acids.

In contrast to the results obtained with B/W mice, the 5-month-old MRL/l mice in our study did not demonstrate an effect of diet on the incidence of lesions in the renal arteries. Aortic lesions were, if anything, higher in the groups fed low-fat diets, and very few mice had lipid-laden lesions. The incidence of vasculitis was higher in the two groups of mice followed to the time of death. More importantly, 43% of the MRL/l mice fed a high-fat, cholesterol-containing diet from 5–6 months to death had atheromatous lesions, whereas none of the mice fed a low-fat diet had such lesions.

Autoimmune mice die of the complications of immune complex deposition. From observations of the non-lymphoproliferative MRL/n mice it is clear that in the absence of active autoimmune disease the high fat diets and the consequent changes in plasma lipid levels were not sufficient to cause either vasculitic or atheromatous lesions. The fact that the life span of MRL/l mice could be so dramatically extended by late advent caloric restriction argues that B-cell hyperactivity is not initiated until months after the T-cell lymphoproliferation is well under way. Likewise, the fact that atherosclerotic lesions were not present at 5 months of age, and yet were quite common at death, argues that these lesions cannot develop until very late in the timetable of the autoimmune condition.

Vascular disease in autoimmune mice has been related to vascular damage caused by circulating immune complexes.[1,2] These complexes are usually cleared from the plasma by macrophages, and macrophages are also involved in lipoprotein clearance and metabolism.[49-51] It is possible that the CICs may compromise the capacity of macrophages to metabolize lipoproteins. As described in the accompanying paper, hypercholesterolemia in MRL mice was also associated with alterations in production of the prostaglandins thromboxane A_2 and PGI_2. Thus, it is possible that altered macrophage function and the changes in prostaglandin synthesis contribute to the diet-induced atherosclerotic vascular disease.

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