

Effects of Cholesterol Loading on Autoimmune MRL-*lpr/lpr* Mice: Susceptibility to Hypercholesterolemia and Aortic Cholesterol Deposition

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ABSTRACT—Autoimmune MRL-*lpr/lpr* (MRL/*l*) mice, with a systemic lupus erythematosus-like disease, were shown to spontaneously develop hyperlipidemia and yet be susceptible to diet-induced hypercholesterolemia and aortic cholesterol deposition. Control animals on a basal diet showed significant increases in the serum total cholesterol, phospholipids, triglycerides, high density lipoprotein (HDL)-cholesterol and lipid peroxide levels, but a significant decline in the serum lecithin:cholesterol acyltransferase (LCAT) activity compared to those of 5-week-old mice. Animals on the high-cholesterol diet showed a rapid rise in serum total cholesterol to a plateau level (800 mg/100 ml) that was approximately 2.5 times higher than that in the control animals on a basal diet. However, the levels of serum triglycerides, HDL-cholesterol and lipid peroxides significantly decreased (by 61%, 23% and 53%, respectively) compared to those of the control animals, whereas LCAT activity and phospholipid level were not affected. The aortic contents of total cholesterol, free cholesterol and cholesteryl ester were significantly higher (by 35%, 36% and 31%, respectively) in animals fed the high-cholesterol diet than the control animals. These findings suggest that MRL/*l* mice are susceptible to diet-induced hypercholesterolemia and aortic cholesterol deposition.

Keywords: Autoimmune, MRL-*lpr/lpr* mice, Hypercholesterolemia, Cholesterol deposition (aortic)

Atherosclerosis is considered to be a reparative response of the vascular wall to injury, having many of the features of the inflammatory response in other tissues (1, 2). Some studies indicate that the incidence of cardiovascular diseases and atherosclerotic manifestations are higher in patients with chronic inflammatory diseases such as rheumatoid arthritis and systemic lupus erythematosus than in controls (3, 4). However, other studies have yielded results contradicting this relationship (5, 6).

Studies with MRL *lpr/lpr* (MRL/*l*) mice, a model of spontaneous lupus-like autoimmune disease, have shown that these animals frequently develop the degenerative vascular disease and myocardial infarction, and that the pathogenesis is associated with the immune complex (7, 8). Atherosclerotic vascular alterations have also been noted to be induced in adjuvant arthritis rats, a model of rheumatoid arthritis (9). Mice are generally considered to be resistant to atherosclerosis and are not usually used as an animal model for atherosclerosis research. An arterial wall injuring agent in addition to a cholesterol-rich diet is required to induce atherosclerotic lesions of the aorta (10). However, certain inbred strains of mice can exhibit

susceptibility to diet-induced atherosclerosis and hyperlipidemia (11–14). In the present study, we attempted to characterize the biochemical aspects in the serum and aorta of MRL/*l* mice fed a high-cholesterol diet. This was examined to elucidate whether the MRL/*l* mice are useful for the study of hypercholesterolemia and atherosclerosis.

MATERIALS AND METHODS

Animals and diets

MRL/*l* mice were bred and maintained in our animal facility from breeding pairs kindly supplied by Research Laboratories, Nippon Shinyaku Co., Ltd. (Kyoto). The mice had been originally obtained from The Jackson Laboratory (Bar Harbor, ME, U.S.A.). ICR mice were obtained from Japan SLC, Inc. (Hamamatsu). They were housed in an air-conditioned room ($23 \pm 2^\circ\text{C}$ and $60 \pm 10\%$ relative humidity) under an artificial 12-hr light/dark cycle (7:00–19:00). All animals used in this experiment were male and 5 weeks old at the start of the experiment. They were given a purified basal diet or a high-cholesterol diet. The basal diet contained 20% casein, 63.2%

sucrose, 10% corn oil, 2% agar, 0.8% vitamin mixture and 4% salt mixture (15). The high-cholesterol diet consisted of the basal diet with 1.5% cholesterol and 0.5% cholic acid in place of an equal amount of sucrose. Each animal was given 5 g of the respective diet daily for 12 weeks. Water was freely available.

Determination of clinical manifestations

Lymph node enlargement was detected by palpating mice biweekly and quantified by an index. Lymph nodes in the cervix and either side of the axilla were graded on a semi-quantitative scale from 0 to 5 according to increasing extent of lymphadenoma: 0, not recognizable by palpation; 1, slightly recognizable by palpation; 2, easily recognizable by palpation; 3, faintly visible to the naked eye; 4, easily visible to the naked eye; 5, prominently visible to the naked eye. The maximum possible score was 15.

Proteinuria was estimated on a 0 to 5 scale by the dipstick method using tetrabromophenol paper (Wako Pure Chemical Ind., Osaka): 0, 0 mg/dl; 1, 10–20 mg/dl; 2, 30 mg/dl; 3, 100 mg/dl; 4, 300 mg/dl; 5, over 1000 mg/dl.

Sample procedures

Blood samples were taken from the ophthalmic vessels under ether anesthesia at biweekly intervals to measure serum cholesterol after the animals had fasted overnight. At the end of the experiment, the mice, after overnight fasting, were killed under ether anesthesia. The serum was separated by centrifugation ($1500 \times g$ for 10 min) and the high-density lipoprotein (HDL) fraction was immediately separated from a portion of the serum by the heparin-manganese precipitation procedure (16). Part of the aorta in the region adjacent to the aortic valve was imbedded in O.C.T. compound (Miles Laboratories, Inc., Naperville, IL, U.S.A.), and the frozen sections were stained with oil red O. The residue of aorta from the arch to the iliac bifurcation was excised and cleaned of periaortal connective tissues and fatty substances, and then it was freeze-dried to a constant weight and lipids were subsequently extracted at 50°C for 20 min with chloroform-methanol (2:1, v/v). The extracts as well as the serum and the HDL fraction were used for lipid determination and enzyme assay. For the histopathological investigation, part of the kidney was fixed in 10% neutral buffer formalin, and the paraffin-embedded sections were stained with hematoxylin and eosin.

Assay of anti-DNA antibodies and rheumatoid factor (RF)

Antibodies to single-stranded DNA (ssDNA) and IgM RF in the serum were assayed by the enzyme-linked immunosorbent assay described by Miyawaki et al. (17).

Microanalysis of cholesterol

Aorta and serum cholesterol levels were determined by the fluoroenzymatic method described previously (18, 19). Aliquots (1/4–1/6) of the aorta extract were dissolved in acetone (100 μ l) containing 2.5 mg of Triton X-100 and evaporated using a centrifugal evaporator. The residues were suspended in 200 μ l of the enzymatic cholesterol reagent, which consisted of 3.6 U cholesteryl ester hydrolase (EC 3.1.1.13, Toyobo Co., Osaka), 1.2 U cholesterol oxidase (EC 1.1.3.6, Oriental Yeast Co., Tokyo), 450 U horseradish peroxidase (EC 1.11.1.7, Seikagaku Kogyo Co., Tokyo), 5 mg homovanillic acid and 20 mg Triton X-100 (Nacalai Tesque, Inc., Kyoto) in 0.1 M Na-Na phosphate buffer (pH 7.0). The contents were thoroughly mixed and incubated at 37°C for 30 min. Following incubation, 2.8 ml of 0.05 N NaOH was added to the reaction mixture, and the fluorescence of each solution was measured. The excitation wavelength was 323 nm, and the emission wavelength was 420 nm. For the determination of the free cholesterol (FC) concentration, only cholesteryl ester hydrolase was removed from the enzymatic cholesterol reagent formulation. To each HDL fraction (10 μ l), 0.15 M disodium ethylenediaminetetraacetate (EDTA) solution (10 μ l) was added in order to prevent the inhibitory action of Mn^{2+} ion on the enzymatic reaction. Cholesterol levels of serum samples (1 μ l) and EDTA-treated HDL fractions (20 μ l) were determined by directly adding the enzymatic cholesterol reagent (200 μ l) described above.

For cholesteryl ester hydrolase, the enzyme from *Pseudomonas sp.* or *Schizopyllum commune* should be used. The enzyme from bovine pancreas is not suitable because it can not completely split cholesteryl ester into cholesterol and fatty acid.

Analysis of other lipids and LCAT activity

Triglycerides were determined by the acetylacetone method of Fletcher (20). Phospholipids were determined by the method of Yoshida (21). LCAT activity was obtained by measuring the rate of FC esterification in the serum by a modified method (18, 19) originally developed by Dieplinger and Kostner (22). The activity was expressed as nmoles of esterified cholesterol per ml of serum per hr. Lipid peroxides were fluorometrically determined by the thiobarbituric acid (TBA) method of Yagi (23).

Statistics

The results obtained were expressed as the mean \pm S.E. of data from 10 mice per group. Student's *t*-test for paired observations, was used to test for significance. However, proteinuria and lymphadenoma scores were assessed by the Wilcoxon rank-sum test.

RESULTS

Twenty MRL/*l* mice were divided into two groups which were given the basal diet (control group) or the high-cholesterol diet (HC group). ICR mice were treated in the same manner for reference.

Changes in body weight

Weight gain in the animals of the control group increased steadily until they were 10-week-old and then remained about the same. The growth rate of the HC group was moderately but significantly inhibited compared to that of the control group throughout the experimental period (Fig. 1). The degree of inhibition was

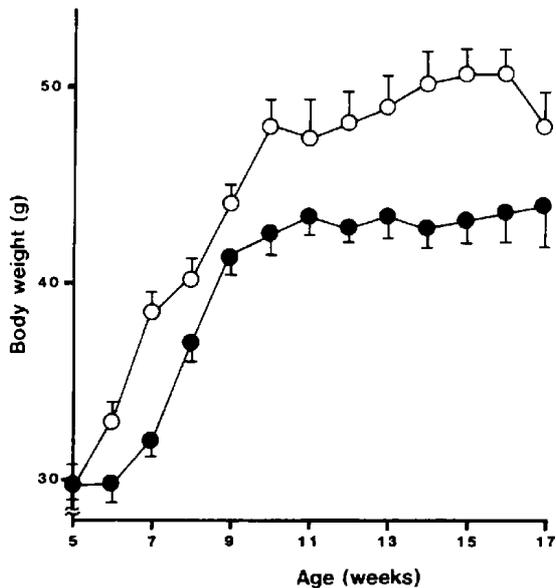


Fig. 1. Changes in body weight in MRL/*l* mice fed the high-cholesterol diet (HC). Diet composition is detailed in Materials and Methods. Each point is the mean \pm S.E. of 10 mice. \circ : Control, \bullet : HC.

comparable to that observed in the experiment of ICR mice under the same condition (data not shown).

Pathologic manifestations

Figure 2 shows the time-related changes in the lymph node enlargement of the cervix and axilla in both groups. Lymphadenopathy gradually developed with age. Dietary cholesterol significantly suppressed it during the whole stage examined. At the end of the experiment, the development of glomerulonephritis was observed histologically, and heavy proteinuria appeared in both groups (Table 1). The serologic abnormalities are also shown in Table 1. The serum levels of anti-ssDNA antibody (IgG) and IgM RF were markedly higher in both groups of animals compared to those of 5-week-old mice.

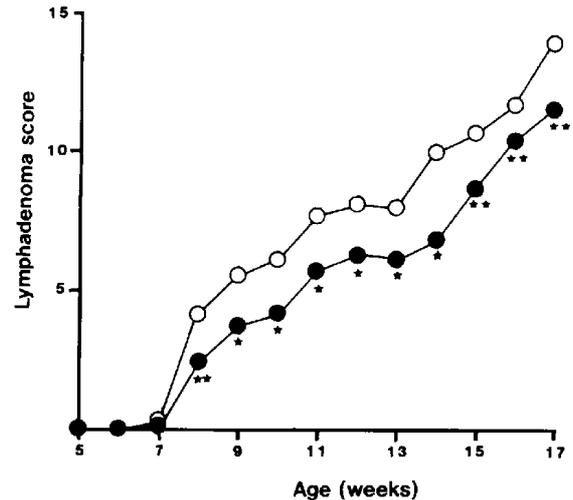


Fig. 2. Changes in lymph node enlargement in MRL/*l* mice fed the high-cholesterol diet (HC). Diet composition is detailed in Materials and Methods. Each point is the mean \pm S.E. of 10 mice. Significant difference from the control, * $P < 0.05$, ** $P < 0.01$. \circ : Control, \bullet : HC.

Table 1. Proteinuria and serologic abnormalities in MRL/*l* mice fed the high-cholesterol diet (HC) for 12 weeks

Age (weeks)	Group	n	Proteinuria (score)	anti-ssDNA	IgM RF
				ELISA value (O.D. unit)	
5		10	1.13 \pm 0.38	0.005 \pm 0.003	0.002 \pm 0.001
17	Control	10	3.38 \pm 0.32**	0.692 \pm 0.223**	0.340 \pm 0.040**
	HC	10	2.88 \pm 0.42	0.668 \pm 0.144	0.325 \pm 0.020

Group composition is detailed in Materials and Methods. n: number of animals. Each value represents the mean \pm S.E. Significant difference from the 5-week-old mice, ** $P < 0.01$. ssDNA: single-stranded DNA, RF: rheumatoid factor, ELISA: enzyme-linked immunosorbent assay.

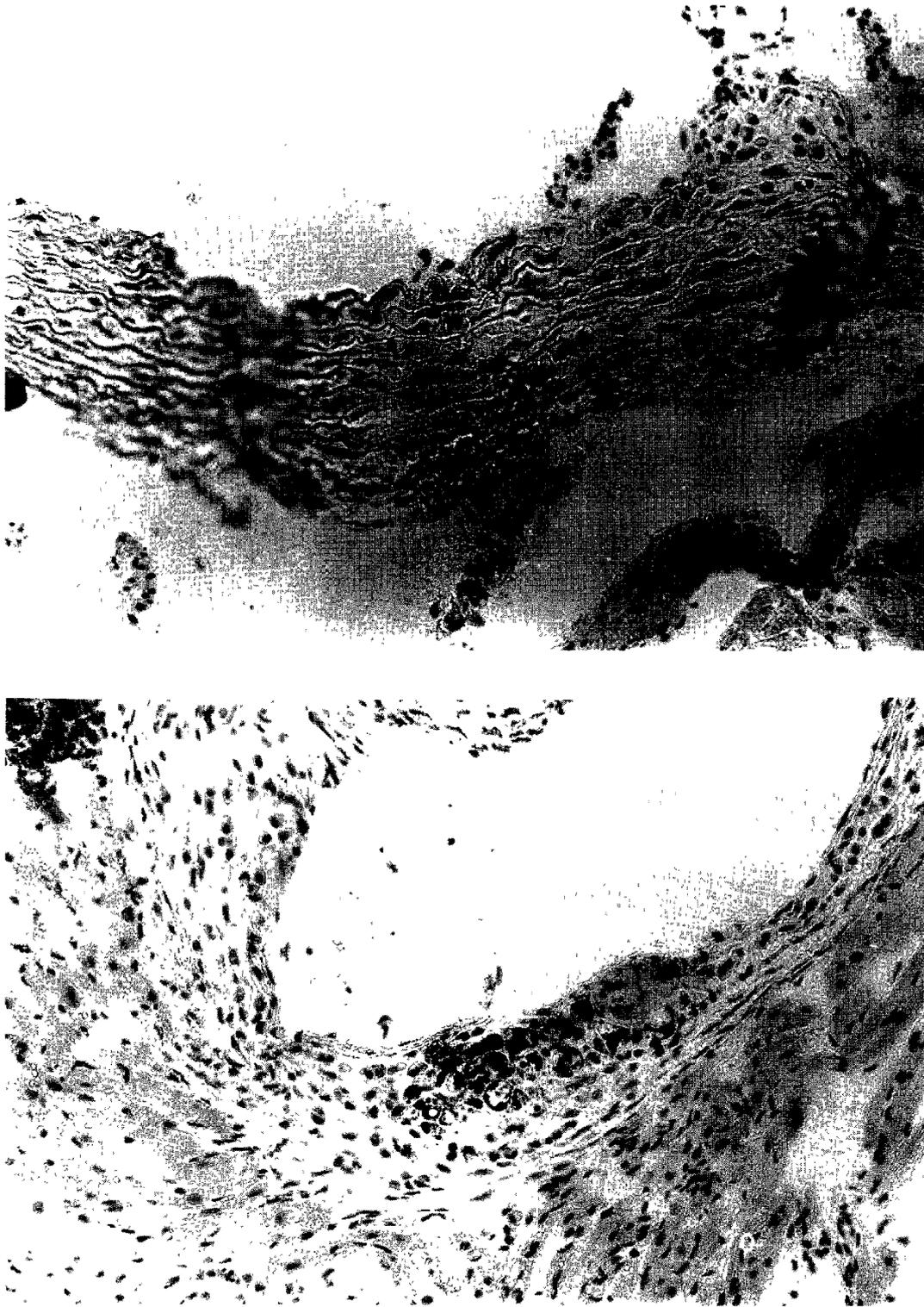


Fig. 3. Histological appearance of an atherosclerotic lesion in the aorta of a MRL/*l* mouse fed the high-cholesterol diet (HC) for 12 weeks. Upper panel: Control group. Lower panel: HC group. Oil red O stain. $\times 250$.

Weights of lymph nodes

At the end of the experiment, the weights of various lymph nodes (cervix, axilla and mesentery nodes) of the control group were markedly higher (74-fold, 34-fold and 39-fold, respectively) than those in the 5-week-old mice. The HC group showed slight decreases in the lymph node weights of the cervix and axilla, while there was a significant increase in that of the mesentery (Table 2).

Histopathological findings

After 12 weeks on the high-cholesterol diet, some of the subendothelial cells of the aorta in the region near the aortic valve showed accumulations of fatty droplets and foam cells (Fig. 3), while the control animals on the basal diet showed no droplets.

Changes in serum total cholesterol level

Figure 4 shows changes in the mean total cholesterol

Table 2. Lymph node weight of MRL/*l* mice fed the high-cholesterol diet (HC) for 12 weeks

Age (weeks)	Group	n	Cervix (mg)	Axilla (mg)	Mesentery (mg)
5		10	11 ± 7	23 ± 3	33 ± 10
17	Control	10	814 ± 189**	790 ± 109**	1282 ± 230**
	HC	10	428 ± 101	615 ± 87	2701 ± 383 [#]

Group composition is detailed in Materials and Methods. n: number of animals. Each value represents the mean ± S.E. Significant difference from the 5-week-old mice, **P < 0.01. Significant difference from the control mice, [#]P < 0.01.

levels of serum from MRL/*l* mice on the high-cholesterol and basal diets. The mean values for the serum total cholesterol level were significantly higher in the HC group compared to the control group throughout 12 weeks. The mice in the control group also showed an elevated mean serum total cholesterol level, that was approximately 100 mg/100 ml higher than the start level, in contrast to ICR mice given the basal diet that remained at a fairly con-

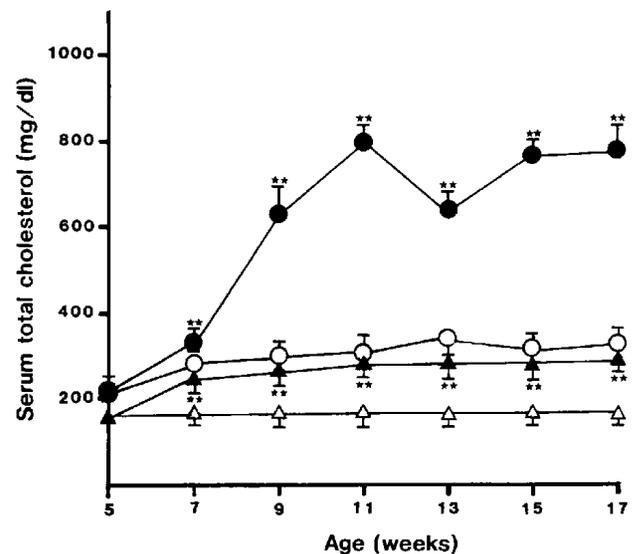


Fig. 4. Changes in serum total cholesterol levels in MRL/*l* mice fed the high-cholesterol diet (HC). ICR mice were used for reference. Diet composition is detailed in Materials and Methods. Each point is the mean ± S.E. of 10 mice. Significant difference from the respective control group, **P < 0.01. ○: MRL (control), ●: MRL (HC), △: ICR (control), ▲: ICR (HC).

Table 3. Serum lipid levels and LCAT activity in MRL/*l* and ICR mice fed the high-cholesterol diet (HC) for 12 weeks

Strain	MRL/ <i>l</i>		ICR			
	Age (weeks)	5	17	17		
Group		Control	HC	Control	HC	
No. of animals		10	10	10	10	
TC (mg/100 ml)		207 ± 7	328 ± 12**	775 ± 63 [#]	157 ± 15	271 ± 13 [#]
CE (mg/100 ml)		154 ± 6	265 ± 29**	611 ± 53 [#]	122 ± 12	199 ± 10 [#]
LPO (nmol/ml)		5.46 ± 0.16	15.38 ± 1.17**	9.13 ± 0.76 [#]	5.48 ± 0.14	2.73 ± 0.18 [#]
PL (mg/100 ml)		214 ± 8	307 ± 18**	281 ± 38	205 ± 14	227 ± 16
TG (mg/100 ml)		79 ± 3	172 ± 9**	66 ± 8 [#]	86 ± 11	45 ± 6 [#]
HDL-TC (mg/100 ml)		144 ± 2	182 ± 7**	141 ± 11 [#]	130 ± 8	78 ± 4 [#]
LCAT (nmol/ml/hr)		59.8 ± 4.7	31.8 ± 4.2**	41.3 ± 10.3	54.0 ± 4.7	22.1 ± 3.4 [#]

Group composition is detailed in Materials and Methods. Each value represents the mean ± S.E. Significant difference from the 5-week-old, **P < 0.01. Significant difference from the respective control group, [#]P < 0.01. TC: total cholesterol, CE: cholesteryl ester, LPO: lipid peroxides, PL: phospholipids, TG: triglycerides, HDL: high density lipoprotein, LCAT: lecithin:cholesterol acyltransferase.

Table 4. Aorta cholesterol levels in MRL/l and ICR mice fed the high-cholesterol diet (HC) for 12 weeks

Strain	MRL/l		ICR		
	5	17	17		
Age (weeks)					
Group	Control		Control	HC	
No. of animals	10	10	10	10	
	mg/g dry weight				
TC	8.20±0.16	8.57±0.24	11.58±0.74 ^{##}	6.25±0.21	6.88±0.25 ^{##}
FC	7.13±0.12	7.66±0.30	10.41±0.63 [#]	5.68±0.13	6.12±0.25 [#]
CE	0.88±0.07	0.91±0.16	1.19±0.28 [#]	0.57±0.04	0.76±0.03

Group composition is detailed in Materials and Methods. Each value represents the mean±S.E. Significant difference from the respective control group, [#]P<0.05, ^{##}P<0.01. TC: total cholesterol, FC: free cholesterol, CE: cholesteryl ester.

stant level (approximately 160 mg/100 ml) throughout the experimental period. Although the serum total cholesterol level in the HC group of ICR mice was significantly higher than that in the control group, the degree of increase by cholesterol feeding in ICR mice was extremely less than that in MRL/l mice.

Serum lipid levels and LCAT activity

The serum lipid levels and LCAT activity in MRL/l mice at the end of the experiment are shown in Table 3. The control group showed significant increases in the serum total cholesterol, cholesteryl ester, lipid peroxide, phospholipid, triglyceride and HDL-cholesterol levels (by 59%, 72%, 173%, 43%, 117% and 26%, respectively) and a significant decline (by 47%) in LCAT activity compared to the 5-week-old mice. The HC group showed further marked increases in the serum total cholesterol, free cholesterol and cholesteryl ester levels (by 136%, 161% and 131%, respectively), but significant decreases in the lipid peroxide, triglyceride and HDL-cholesterol levels (by 41%, 62% and 23%, respectively) compared to the control group. However, the LCAT activity and phospholipid levels were not influenced by the cholesterol feeding. Similar changes in lipid levels were observed in ICR mice except for the LCAT activity.

Aortic cholesterol contents

As shown in Table 4, in MRL/l mice, the aortic contents of total cholesterol, free cholesterol and cholesteryl ester were significantly higher in the HC group than in the control group (35%, 36% and 31%, respectively). The aortic cholesterol contents of the control group were comparable to those of the 5-week-old mice. The ICR mice given a high-cholesterol diet showed only a slight increase (10%) in the aortic cholesterol contents compared to the control animals given a basal diet.

DISCUSSION

This study indicates that MRL/l mice spontaneously develop hyperlipidemia as well as massive lymphadenopathy with age, and yet they are susceptible to diet-induced hypercholesterolemia and thereby also susceptible to arterial cholesterol deposition.

The serum cholesterol level in mice on a standard diet without cholesterol supplement generally remains unchanged over several months, even in mice genetically susceptible to atherosclerosis (12–14). In this study, we also observed that when ICR mice, a strain resistant to cholesterol feeding, was used for reference and given a basal diet, the serum cholesterol level was fairly constant throughout the experimental period, from 5 to 17 weeks of age. On the other hand, the serum cholesterol level in MRL/l mice maintained on a basal diet gradually increased with age and finally became approximately 1.6 times higher than that at the start of the experiment (5-week-old mice). The serum levels of other lipids, such as phospholipids, triglycerides, HDL-cholesterol and lipid peroxides, also significantly increased. It is interesting that the lipid peroxide levels increased in the serum taken from MRL/l mice. Increased superoxide generation and the consequently augmented lipid peroxidation reaction play an important role in the pathogenesis of autoimmune diseases including rheumatoid arthritis and systemic lupus erythematosus (24, 25). Furthermore, in MRL/l mice, the superoxide release of macrophages is remarkably elevated compared to that of macrophages in MRL/n mice (26). Thus, the increased lipid peroxide levels in MRL/l mice may result from autoimmune abnormalities which develop in MRL/l mice.

In this study, we also observed a marked decline of LCAT activity in MRL/l mice that developed autoimmune disease. Attention was focused on the relationship

between autoimmune diseases and cytokines such as interleukin-1 and interleukin-6 (27–30). We have previously reported that adjuvant arthritic rats (31) show a marked decline in LCAT activity. A similar decline is found in rats given various cytokines intraperitoneally (32). The lupus lesions in MRL// mice are characterized by deposition of immunocomplexes and accumulation of macrophages (26), which can release various cytokines. These findings suggest that the observed decline of LCAT activity in MRL// mice may be due to the action of cytokines produced during the progression of the autoimmune disease.

It is not clear whether genetic control is involved in the hyperlipidemia found in MRL// mice. It is possible that hyperlipidemia may result from glomerulonephritis which develops in MRL// mice (33), since the nephrotic syndrome is known to be closely related to hyperlipidemia (34, 35). However, hyperlipidemia in MRL// mice was not ameliorated by administration of cyclophosphamide, an immunosuppressive agent, although glomerulonephritis and the serological parameters of the autoimmune disease were prevented (data not shown). Therefore, hyperlipidemia in MRL// mice is probably not the secondary change following the renal lesions, but a phenomenon involving specific genetic control.

MRL// mice which had been fed a high-cholesterol diet also showed sustained and marked hypercholesterolemia, in which the cholesterol level (approximately 800 mg/100 ml) was comparable to that obtained in mice from a strain genetically susceptible to hypercholesterolemia, such as C57BL/6J and C57BR/cdj (12–14), although mice from the resistant strain as well as ICR have shown only about two-fold elevation under the same conditions (10, 12, 13, 19). These data indicate that MRL// mice would be one of the strains susceptible to diet-induced hypercholesterolemia. On the other hand, the levels of serum triglycerides, HDL-cholesterol and lipid peroxides significantly decreased, as compared to those of the control animals. Such lipid changes are commonly observed in other mice maintained on a high-cholesterol diet (10, 19), but the mechanism is at present unknown.

To the best of our knowledge, this is the first report of biochemical determination of cholesterol contents in the aorta of MRL// mice. Microanalysis of tissue cholesterol by a fluorometric enzymatic method made it possible to determine the free cholesterol and cholesteryl ester contents in mouse aorta. In this study, we observed a marked increase (by 35%) in the aortic total cholesterol content in MRL// mice fed a high-cholesterol diet for 12 weeks, compared to that in the control mice fed a basal diet. The older, control mice (17-week-old) had the same cholesterol content in the aorta as the young mice (5-week-old), in spite of the increased serum cholesterol levels. When con-

ventionally used mice such as ICR and C57BL/6Cr are maintained on a high-cholesterol diet for 10 or 14 weeks, the increase rate of cholesterol content in the aorta is at most about 10% (10, 19), although it reaches over 40% under severe conditions when the animals are maintained on a high-cholesterol diet supplemented with β -aminopropionitrile (10). Therefore, such cholesterol deposition in MRL// mice may be associated with the immune response and hypercholesterolemia, since MRL// mice display a necrotizing polyarteritis which develops secondarily to high levels of autoantibodies with a concomitant rise in immune complexes (8, 36).

It is well known that cholesteryl ester predominantly accumulates in atherosclerotic lesions (37). In this study, however, aortic lipid deposition in MRL// mice by cholesterol feeding was characterized by an accumulation of free cholesterol. Although such a paradoxical phenomenon is not yet known, it is possible that in the aorta of MRL// mice, acylcoenzyme A: cholesterol acyltransferase, an enzyme catalyzing an esterification of free cholesterol, is inhibited by cholesterol feeding. Investigation of this possibility must be the subject of further studies. Since MRL// mice are susceptible to dietary cholesterol and are relatively easy to obtain and inbreed through brother-sister mating, they may be usable for the study of hypercholesterolemia and atherosclerosis, as well as systemic lupus erythematosus.

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