Immune-Complex–Mediated Vasculitis Increases Coronary Artery Lipid Accumulation in Autoimmune-Prone MRL Mice

Jian-Hua Qiao, Lawrence W. Castellani, Michael C. Fishbein, and Aldons J. Lusis

MRL/lpr mice develop severe autoimmune disease and vasculitis by 5 months of age, whereas congenic strain MRL/n mice exhibit much milder vasculitis with a later age of onset. When maintained on a high-fat, high-cholesterol (atherogenic) diet, strain MRL/lpr mice exhibited a striking deposition of lipid in both the large and small coronary arteries, whereas strain MRL/n mice exhibited very little lipid accumulation. Neither strain exhibited lipid accumulation on a low-fat chow diet. The atherogenic diet induced hyperlipidemia in both strains, but surprisingly the levels of atherogenic apolipoprotein B–containing lipoproteins were much lower in MRL/lpr mice. Immunohistochemical studies revealed that immune complexes (immunoglobulins G and M), T and B lymphocytes, macrophages, granulocytes, apolipoprotein B, and serum amyloid A proteins were present in the walls of the coronary arteries that had vasculitis and lipid accumulation. By 6–7 months of age, MRL/lpr mice had a higher incidence of myocardial infarction in the atherogenic diet group (53%) compared with the chow group (14%), whereas MRL/n mice exhibited no myocardial infarction on either diet. These results suggest important interactions between vasculitis, hyperlipidemia, and arterial lipid accumulation. They support the concept that injury to the vessel wall in immune-complex–mediated vasculitis increases lipid deposition in the presence of hyperlipidemia. (Arteriosclerosis and Thrombosis 1993;13:932–943)

KEY WORDS • vasculitis • systemic lupus erythematosus • immunohistochemistry • coronary atherosclerosis • myocardial infarction • plasma lipoproteins • cholesterol

Two congenic inbred MRL mouse substrains, MRL/MpJ-lpr/lpr (MRL/lpr) and MRL/MpJ-+/+ (MRL/n), differ with respect to the presence of an autosomal mutant gene lpr. 1 MRL/lpr mice, which carry the lpr locus, have phenotypic characteristics of systemic lupus erythematosus (SLE), a human autoimmune connective-tissue disease. The characteristics include hypergamaglobulinemia, anti-DNA antibodies, rheumatoid factor, circulating immune complexes, massive lymphadenopathy, glomerulonephritis, polyarteritis, and vasculitis. 1-6 Mice of the MRL/n substrain lacking the lpr gene show no generalized lymphoproliferation, have a milder manifestation of autoimmune disease later in life, and develop far fewer vascular lesions. 1-2 Studies of lpr mice (MRL/lpr and other strains of mice that carry the lpr locus) suggest that there is a defect in the negative selection of self-reactive T lymphocytes in the thymus. 7-9 Excessive numbers of self-reactive T lymphocytes that are released into peripheral organs appear to contribute to the autoimmune disease, since neonatal thymectomy prevents the appearance of the lymphoproliferative disease in MRL/lpr mice. 1,6 Bone marrow transplantation studies indicate that the lpr mutation causes an intrinsic T-lymphocyte abnormality responsible for lymphadenopathy and autoantibody production. 10 Recent studies have shown that the lymphoproliferation disorder in lpr mice is explained by a defect in the Fas antigen that mediates apoptosis, a type of cell death that plays an important role in early development and growth of normal adult tissues. 11-13 MRL mice exhibit both degenerative vascular disease (DVD) and necrotizing polyarteritis (NPA). 1-4,14-16 The vasculitis occurs predominantly in small and mediumsized arteries of a variety of tissues, including the kidney, genital organs, and heart. NPA involves two distinct histopathological types: 1) neutrophilic, immune-complex–mediated vasculitis and 2) mononuclear-cell vasculitis. 17,18 The pathological features of NPA have been extensively studied. 17-23 In the acute stages of NPA, polymorphonuclear leukocytes infiltrate all layers of the vessel wall and the perivascular areas. Mononuclear-cell infiltration follows as these lesions become more chronic. 15,17,18 The development of NPA is associated with high levels of circulating immune complexes that are deposited in the vessel walls. DVD, on the other hand, lacks the acute pathological components. It generally involves minimal local mononuclear-cell infiltration and occurs predominantly in the coronary arteri-
ies, where it is associated with thrombosis and myocardial infarction.\textsuperscript{14-16} In autoimmune MRL/lpr mice, the incidence of DVD is low compared with that of NPA.\textsuperscript{14-16}

In this article, we report a detailed histological study of coronary and aortic lesions in MRL mice maintained on a low-fat chow diet as well as a high-fat, high-cholesterol diet. The latter diet induces early aortic atherosclerotic lesions (fatty streaks) in certain genetically susceptible inbred strains of mice, such as C57BL/6j.\textsuperscript{24-29} Previous work with MRL/lpr mice has shown that a similar high-fat, high-cholesterol diet increases the incidence of atherosclerotic lesions of intrarenal and aortic branch arteries.\textsuperscript{30} Our results reveal significant interactions between the development of vasculitis and diet-induced hyperlipidemia. They demonstrate that the vasculitis in MRL/lpr mice is associated with lipid and lipoprotein accumulation in the vessel walls. This is accompanied by an increase in the frequency of myocardial infarction. The results suggest that arterial lipid accumulation resulting from immune-complex-mediated vascular injury may be a contributing factor to the premature coronary atherosclerosis and myocardial infarction observed in patients with SLE.\textsuperscript{31-36}

Methods

Mice and Diets

Male and female MRL/MPJ-lpr/lpr (MRL/lpr) and MRL/MPJ-+/+ (MRL/n) mice were obtained from The Jackson Laboratory (Bar Harbor, Me.). Three to five mice were housed per cage and maintained in a temperature-controlled room on a 12-hour light/dark cycle. Mice were fasted overnight before bleeding or sacrifice.

At 3 months of age, the animals were assigned to one of two dietary groups and were maintained on the diet for 15 weeks. The atherogenic diet (Food-Tek, Inc., Morris Plains, N.J.) contained, by weight, 7.5% cocoa butter, 1.25% cholesterol, and 0.5% cholic acid, with a total fat content of 15%. This diet induces hyperlipidemia, resulting in aortic fatty streak lesion formation in certain strains of mice, including C57BL/6j.\textsuperscript{24-29} The regular chow diet (Purina No. 5005) contained 4% fat.

Analysis of Plasma Samples

Mice were bled retro-orbitally under isoflurane anesthesia (Forane, Anaquest, Madison, Wis.) at 0, 5, and 15 weeks of the experiment. Blood was collected directly through heparinized capillary tubes (Fisher Scientific) into EDTA-treated Microtainer tubes (Becton Dickinson) and plasma was separated by centrifugation. Cholesterol and triglyceride (TG) assays were performed in 96-well microtiter plates (Costar No. 3598) using a Biomek 1000 Automated Laboratory Workstation (Beckman). Plasma lipids were determined as described,\textsuperscript{37} including total cholesterol, TG, free fatty acids (FFAs), and glycerol. High density lipoprotein cholesterol (HDL-C) was measured after the addition of heparin and manganese to precipitate apolipoprotein (apo) B-containing very low density lipoproteins (VLDLs) and low density lipoproteins (LDLs), followed by centrifugation at 15,000g for 15 minutes to pellet the precipitate. The supernatants were taken for determination of HDL-C. Plasma lipoproteins were also isolated by gel filtration chromatography (Pharma-LKB fast protein liquid chromatography system). One hundred microliters of pooled plasma (from four to six animals) was chromatographed on a Superox 6 column as described,\textsuperscript{38} and the cholesterol content of each 0.5-mL fraction was determined.

<table>
<thead>
<tr>
<th>Table 1. Primary Antibodies Used in This Study</th>
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<tbody>
<tr>
<td><strong>Antibody</strong></td>
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<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>Mac-1</td>
</tr>
<tr>
<td>CD4</td>
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<tr>
<td>CD8</td>
</tr>
<tr>
<td>CD23</td>
</tr>
<tr>
<td>Gr-1</td>
</tr>
<tr>
<td>IgG</td>
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<tr>
<td>IgM</td>
</tr>
<tr>
<td>SAA1 and SAA2</td>
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<tr>
<td>SAA3</td>
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<tr>
<td>ApoB</td>
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Ig, immunoglobulin.

Dietary-Induced Atherosclerosis in MRL Mice

At 3 months of age, the animals were assigned to one of two dietary groups and were maintained on the diet for 15 weeks. The atherogenic diet (Food-Tek, Inc., Morris Plains, N.J.) contained, by weight, 7.5% cocoa butter, 1.25% cholesterol, and 0.5% cholic acid, with a total fat content of 15%. This diet induces hyperlipidemia, resulting in aortic fatty streak lesion formation in certain strains of mice, including C57BL/6j.\textsuperscript{24-29} The regular chow diet (Purina No. 5005) contained 4% fat.

<table>
<thead>
<tr>
<th>Table 2. Plasma and Lipoprotein Lipids*</th>
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<tbody>
<tr>
<td><strong>Strain (diet)/sex (No.)</strong></td>
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<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>MRL/lpr (chow)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>MRL/n (chow)</td>
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<tr>
<td></td>
</tr>
<tr>
<td>MRL/lpr (HF)</td>
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<tr>
<td></td>
</tr>
<tr>
<td>MRL/n (HF)</td>
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*Total plasma cholesterol and triglyceride levels (expressed in milligrams per deciliter) were determined at the start of the experiment (12 weeks of age) and after 15 weeks on either the chow or atherogenic (HF) diet (27 weeks of age). High-density lipoproteins (HDLs) were isolated by precipitating apolipoprotein B-containing lipoproteins from plasma with heparin-manganese, followed by centrifugation. Cholesterol assays were then performed on the supernatants as a measure of HDL cholesterol levels. The values are the mean±SEM.

†Indicates significant differences between males and females of the same age and strain and on the same diet, p<0.05.

‡Indicates differences between the chow and atherogenic diet of the same sex, strain, and age, p<0.05.

§Indicates differences between MRL/n and MRL/lpr of the same sex and on the same diet, p<0.05.
FIGURE 1. Separation of plasma lipoproteins of MRL mice by gel filtration chromatography. Plasma (100 μl, pooled from four to six animals of each group) was chromatographed on a Superose 6 column as described,33 and the cholesterol content of each fraction (0.5 ml) was determined. Panel A: Strain MRL/n (—) and strain MRL/lpr (▬) mice maintained on a low-fat chow diet. All mice were females 6–7 months of age. Panel B: Strain MRL/n (—) and strain MRL/lpr (▬) mice maintained for 15 weeks on a high-fat, high-cholesterol, atherogenic diet. All mice were females 6–7 months of age. The fractions in which very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) elute are indicated.38

Histopathological Studies
Animals were killed by cervical dislocation. At autopsy, the heart and proximal aorta were dissected. Each excised heart was washed once in phosphate-buffered saline to remove the blood from the heart chambers and the lumen of the aorta. The basal portion of the heart and root of the aorta were embedded in OCT compound and frozen on dry ice. Serial 10-μm-thick cryosections were collected on poly-D-lysine-coated slides and stored at −70°C for lipid staining and immunohistochemical studies (described below). The apex of the heart and other tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned (4-μm-thick) for routine histological examination with hematoxylin and eosin staining.

Evaluation of Atherosclerotic Lesions
Each heart was frozen and sectioned as described above for quantitative evaluation of atherosclerotic lesion formation in the root of the aorta. The preparation for sectioning and the plane at which the first cut was made have been described by Paigen et al.24 The sectioned region began at the aortic root and continued toward the aortic arch for a distance of approximately

TABLE 3. Fatty Streak Lesion Formation in the Root of the Aorta*

<table>
<thead>
<tr>
<th>Diet/sex</th>
<th>MRL/lpr</th>
<th>MRL/n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lesion area (μm²/section)</td>
<td></td>
</tr>
<tr>
<td>HF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>3,184±1,511 (7)</td>
<td>2,085±1,983 (4)</td>
</tr>
<tr>
<td>Female</td>
<td>2,576±1,011 (7)</td>
<td>7,371±2,910 (5)</td>
</tr>
<tr>
<td>Total</td>
<td>2,880±877 (14)†</td>
<td>5,022±1,968 (9)‡</td>
</tr>
<tr>
<td>Chow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0 (4)</td>
<td>95±95 (5)</td>
</tr>
<tr>
<td>Female</td>
<td>102±38 (3)</td>
<td>184±184 (4)</td>
</tr>
<tr>
<td>Total</td>
<td>58±29 (7)†</td>
<td>134±91 (9)‡</td>
</tr>
</tbody>
</table>

The values are the mean±SEM for (n) animals.
*Mice were fed a high-fat, high-cholesterol (HF) diet for 10–15 weeks.
†Differences in lesions were significant at p<0.03.
‡Differences in lesions were significant at p<0.01.
400 μm. Every other 10-μm section was collected, stained with oil red O and hematoxylin, and counterstained with fast green FCF. Each of these sections was reviewed by light microscopy and then evaluated quantitatively. The cross-sectional area of lipid-containing lesions was determined by using a microscope eyepiece grid (20x20-grid disc No. 478, AO Scientific Instruments, Buffalo, N.Y.). The areas of all lesions in each section were added together to give a total lesion area per section.

For a quantitative assessment of atherosclerotic lesions in coronary arteries, every fifth 10-μm heart section was collected from the lower portion of the heart, the site of the first cut, to the aortic root as mentioned above, and stained with oil red O. Main coronary arteries and major branches, which were usually around the root of the aorta (near the ostium) or the epicardium were considered as large arteries (diameter =150–250 μm) and their intramyocardial ramifications as medium-sized or small arteries (diameter =75 μm or less). The cross sections of these coronary arteries could be identified clearly under the light microscope. Five cross sections of each mouse heart were evaluated. The total number of cross sections of coronary arteries and the number of affected coronary arteries (vasculitis and/or lipid accumulation) in each mouse heart cross section was counted. The size of lipid-containing lesions was determined by using the microscope grid, as described above.

Immunohistochemical Staining

To further study the characteristics of vasculitis in MRL mice fed the atherogenic diet, the following antibodies were used: 1) monoclonal rat anti-mouse Mac-1 antigen (Boehringer Mannheim Biochemicals, Indianapolis, Ind.); 2) monoclonal rat anti-mouse CD4 (GIBCO BRL); 3) monoclonal rat anti-mouse CD8 (GIBCO BRL); 4) monoclonal rat anti-mouse immunoglobulin (Ig) E Fc receptor (CD23; PharMingen, San Diego, Calif.); 5) monoclonal rat anti-mouse granulocyte (Gr-1) (PharMingen); 6) monoclonal rat anti-mouse IgG (γ-chain; ZYMED Laboratories, Inc., San Francisco, Calif.); 7) monoclonal rat anti-mouse IgM (μ-chain; ZYMED Laboratories); 8) affinity-purified polyclonal rabbit anti-human serum amyloid A proteins (SAA1 and SAA2) that cross-react well against mouse SAA3 (a gift of Dr. F.C. deBeer); and 10) monospecific polyclonal rabbit anti-rat apoB.26 The specificity and optimal dilutions of these antibodies are summarized in Table 1. The avidin-biotinylated peroxidase system used for immunohistochemical staining has been described by Mehrabian et al.26 In brief, 10-μm-thick cryostat sections were fixed in acetone, incubated with primary antibodies, and then incubated with biotinylated, affinity-purified anti-lg and avidin DH/ biotinylated horseradish peroxidase H complex (ABC kit, Vector, Burlingame, Calif.). Antibody binding was visualized with a peroxidase chromogen kit (AEC; Biomedica Corp., Foster City, Calif.). Controls included omission of primary antibody and use of nonimmune sera, as well as use of frozen sections of heart tissues from MRL/n and C57BL/6J chow-fed mice. The slides were counterstained with hematoxylin.

Statistical Analysis

Data analysis was performed using STATVIEW (Student's t test, nonparametric Mann-Whitney U test, χ2 analysis, and analysis of variance) software for the Macintosh.

**Results**

**Plasma Lipoprotein Profiles**

Total plasma cholesterol and TG levels, as well as cholesterol content of the various lipoprotein fractions, were determined in MRL/n and MRL/lpr mice maintained on either a chow diet or a high-fat, high-cholesterol (atherogenic) diet. Table 2 shows the levels of lipids in total plasma and in HDL obtained after heparin-manganese precipitation of lipoproteins containing apoB. Additionally, lipoproteins in pooled plasma samples from females of each experimental group were isolated by gel filtration chromatography (Figure 1). On a chow diet, the majority of the cholesterol was contained in the HDL fraction, and the levels of LDL and VLDL were relatively low. This profile is similar to that observed in other inbred strains; except that the HDL-C levels were unusually high (HDL-C levels in most strains range from 50 to 70 mg/dL).29 The distributions of cholesterol between HDL and LDL + VLDL, as determined by gel filtration and heparin-manganese precipitation, were qualitatively similar, although some quantitative differences were observed. These differences were most likely due to the fact that under certain conditions, heparin-manganese treatment can also precipitate some apoE-containing lipoproteins in addition to the apoB-containing lipoproteins. In both strains, challenge with the atherogenic diet resulted in increases in the levels of LDL, VLDL, and cholesterol-rich particles intermediate in size between the two (Figure 1). Interestingly, the levels of these lipoproteins were substantially higher in MRL/n than in MRL/lpr mice (Figure 1). Since the two strains have a similar genetic background, this finding suggests that the accelerated autoimmune disease may significantly affect lipoprotein metabolism. Plasma FFA and glycerol levels in both MRL/n and MRL/lpr mice were similar to those observed in other mouse strains. Lipid values obtained after 5 weeks on the atherogenic diet showed the same sex-, strain-, and diet-dependent differences observed after 15 weeks (data not shown).
FIGURE 3. Lipid accumulation in coronary arteries exhibiting vasculitis in MRL/lpr mice fed the atherogenic diet for 15 weeks. Panel A: Section showing the aorta (A) and ostium (*) of a proximal coronary artery with necrotizing polyarteritis (NPA) and lipid accumulation. Original magnification, ×10. Panel B: A large coronary artery exhibiting lipid accumulation in three fourths of the circumference. Original magnification, ×10. Panel C: A medium-sized coronary artery showing NPA with circumferential and concentric lipid-containing lesions. Original magnification, ×50. Panel D: A large coronary artery exhibiting degenerative vascular disease (DVD) with segmental and eccentric lipid accumulation. Original magnification, ×100. Panel E: A medium-sized coronary artery exhibiting DVD and an organized mural thrombus (T); some lipid accumulation was present in the media. Original magnification, ×100. Panel F: A small coronary artery with circumferential, intimal, and medial lipid deposition; the lumen was nearly occluded by cellular infiltration and lipid accumulation. Original magnification, ×100. All slides were stained with oil red O, hematoxylin, and fast green.
Table 5. Lipid Accumulation in Coronary Arteries*

<table>
<thead>
<tr>
<th>Diet/sex</th>
<th>MRL/lpr</th>
<th>MRL/n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HF</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>5,480±2,617 (7)</td>
<td>672±413 (4)</td>
</tr>
<tr>
<td>Female</td>
<td>6,839±3,591 (7)</td>
<td>68±44 (5)</td>
</tr>
<tr>
<td>Total</td>
<td>6,160±2,143 (14)</td>
<td>336±200 (9)</td>
</tr>
<tr>
<td><strong>Chow</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0 (4)</td>
<td>25±25 (5)</td>
</tr>
<tr>
<td>Female</td>
<td>0 (3)</td>
<td>0±0 (4)</td>
</tr>
<tr>
<td>Total</td>
<td>0 (7)</td>
<td>14±14 (9)</td>
</tr>
</tbody>
</table>

The values are the mean±SEM for (n) animals.

* Mice were fed a high-fat, high-cholesterol (HF) diet for 10-15 weeks.
† High-fat diet vs. chow diet, p<0.01.
‡ MRL/lpr vs. MRL/n, p<0.01.
§ MRL/lpr females vs. MRL/n females, p<0.03.

Atherosclerotic Lesions in the Aorta

Previous studies have shown that atherosclerosis-susceptible strains of mice, such as C57BL/6J, develop fatty streak lesions in the proximal regions of the ascending aorta in response to an atherogenic diet. We quantified the lesion formation within the proximal regions of the ascending aorta and found lipid accumulation in this region in both MRL/lpr and MRL/n strains after 15 weeks on the atherogenic diet (Figure 2). Few or no lesions were found in either strain on the chow diet. The lesion area (square microns per section) for each strain are shown in Table 3. The difference in lesion development on the atherogenic versus the chow diet was significant (MRL/n mice, p<0.01; MRL/lpr, p<0.01). On the atherogenic diet, MRL/n mice developed more severe fatty streak lesions than MRL/lpr mice (5,022±1,968 versus 2,880±877 μm²/section). Previous studies have revealed that female mice are more susceptible to aortic lesion development than male mice. The difference in lesion development in female mice of the MRL strains with several well-characterized, nonautoimmune-susceptible and -resistant inbred strains. MRL/n mice were relatively susceptible, with lesion development approaching that of the highly susceptible C57BL/6J strain. MRL/lpr mice, on the other hand, resembled the relatively resistant BALB/cJ strain.

Atherosclerotic Lesions in the Coronary Arteries

In MRL/lpr mice fed the atherogenic diet, lipid accumulation occurred frequently in both distal (small and medium-sized) coronary arteries (Figure 3F) and proximal (large) coronary arteries (Figure 3A). The media of the artery was the most frequent site for lipid accumulation (Figure 3). Lipid deposition could be found in all three layers of artery and in areas of surrounding necrotic or degenerative myocytes (Figure 4A). In the proximal coronary arteries, there were two types of lipid-containing lesions: segmental or eccentric (the artery usually had DVD; Figure 3D), and circumferential or concentric (the artery usually had NPA; Figure 3C). Complete occlusion of the small arteries as a result of lipid accumulation, cellular proliferation, and thrombus formation was common (Figure 3F).

Vasculitis and Myocardial Infarction

There was no light microscopic evidence of severe coronary vasculitis in MRL/n mice. In contrast, all MRL/lpr mice (both chow and atherogenic diet groups) had severe coronary vasculitis (Figure 3). Vasculitis usually involved multiple coronary arteries, and the various stages (acute, subacute, and chronic) of vasculitis often occurred within the same heart. Both DVD (e.g., Figures 3D and 3E) and NPA (e.g., Figures 3C and 3F) could occur in the same heart or even in the same coronary artery. The occurrence of coronary vasculitis in MRL/lpr mice is shown in Table 6. There was a higher occurrence of coronary vasculitis in the atherogenic diet group than the chow group. The proximal (large) coronary arteries (e.g., Figures 3A, 3B, and 3D) had a relatively lower occurrence of vasculitis than distal (medium- and small-sized) coronary arteries (e.g., Figures 3C, 3E, and 3F). Coronary arteries that had vasculitis usually had lipid-containing lesions in the same vessel wall (Figure 3), and the severity of lipid accumulation was proportional to the occurrence of coronary vasculitis in the atherogenic diet group (Tables 5 and 6). However, in the chow group, no lipid accumulation occurred within the walls of coronary arteries that had severe vasculitis (data not shown). Cellular proliferation, resulting in the narrowing and obliteration of the lumen of coronary arteries, was a common observation in both chow and atherogenic diet groups of MRL/lpr mice (Figures 3F and 4A). As reported previously, thrombi were usually present in the coronary arteries that had DVD (Figure 3E). Lipid accumulation occurred in both DVD and NPA, although NPA had the most severe lipid-containing lesions (e.g., compare Figures 3C [NPA] and 3E [DVD]).

Eight of 15 MRL/lpr mice (53%) fed the atherogenic diet had myocardial infarcts (single, multiple, chronic, and acute). These myocardial infarcts were observed in the walls of the right ventricle (7/8), left ventricle (5/8), and papillary muscle (1/8). Occluded coronary arteries that had vasculitis and/or lipid-containing lesions were found frequently within or near the infarcts. Only one MRL/lpr mouse in the chow group showed evidence of myocardial infarction (Table 7). The other type of lesions relating to myocardial infarction were degenerative or necrotic loci, which usually involved two or three myocytes (Figure 4A). There was a high incidence (10/15, 67%) of this kind of lesion in MRL/lpr mice fed the atherogenic diet. Degenerative or necrotic loci, which could be secondary to the obliteration of small
coronary arteries, were usually found in multiple areas involving walls of both the left and right ventricles. At sacrifice (about 27 weeks of age), MRL/lpr mice in the atherogenic group had a higher mortality than those in the chow group (Table 7).

**Immunohistochemical Studies**

Monoclonal and polyclonal antibodies were used to examine the cellular and protein composition of coronary lesions in MRL/lpr mice that had been maintained on the atherogenic diet for 15 weeks. The presence of
epitopes was visualized in 10-μm-thick cryostat sections by using an avidin-biotinylated peroxidase system.26

It has been reported that various Ig classes (IgM, IgG1, IgG2, and IgG3) are found in the renal pelvic vessels and coronary arteries of MRL/lpr mice exhibiting evidence of NPA.1415 Using immunocytochemical methods, we previously reported positive staining for immunoglobulin (Ig) M. Original magnification, ×50. Panel E: IgG-positive staining in the same medium-sized vessel. Original magnification, ×100. The inset shows positive staining for IgM in fatty streak lesions in the aortic wall. Original magnification, ×50. Slides for immunohistochemical studies were counterstained with hematoxylin. Panel A was stained with oil red O, hematoxylin, and fast green.

T lymphocytes have been reported in human aortic and coronary intimas and in atherosclerotic lesions of cholesterol-fed rabbits, suggesting a possible role in atherogenesis.4142 In the course of autoimmune diseases, along with hyperfunction and polyclonal activation of B cells, abnormalities of regulatory T cells also seem to participate as primary or accessory factors of the immunopathological process. T-cell number and nonspecific T-helper function are elevated in MRL/lpr mice.6 To examine the distribution of lymphocytes in the lipid-containing lesions of coronary arteries that had vasculitis, we stained frozen sections with monoclonal antibodies to T-cell surface antigens (CD4 and CD8) and a B-cell surface antigen (CD23). Although most of the infiltrated cells in NPA were CD4- and CD8-negative, both CD4 and CD8 T-cell subsets were present in coronary vessels at sites of vasculitis or in the perivascular area (data not shown). CD4- and CD8-positive-staining cells were usually located in the same area, with CD4-positive-staining cells generally being more numerous. CD4- and CD8-positive cells were also present in aortic fatty streak lesions and the loci of degenerative or necrotic myocytes (data not shown). Positive staining of B cells was found less frequently than that of T cells. B cells were usually present in the area of cellular infiltration in the myocardium and connective heart tissue. Occasionally, B cells were also found in coronaries that had vasculitis. Both T and B lymphocytes were present in the regions of epicarditis (Figure 5F).

Early atherosclerotic lesions are characterized by the accumulation of monocytes/macrophages and lipoproteins in the arterial intima of both humans and experimental animals.2642-45 Using immunocytochemical methods, we previously reported positive staining for Mac-1 and apoB in the region of atherosclerotic lesions in the aortic root of C57BL/6J mice fed an atherogenic diet for 15-22 weeks.26 Mac-1 antibody binds to antigens on mouse monocytes/macrophages and neutrophils. In agreement with these results, we found Mac-1-positive cells in the aorta in the same sites that exhibited lipid-containing lesions (data not shown). Numerous Mac-1-positive cells were observed within and around coronary arteries that had vasculitis and lipid-containing lesions (Figure 4B). Mac-1-positive cells were also found in areas of cellular proliferation within the myocardium and epicardium (Figure 5C). Positive staining of granulocytes was usually found in the same areas of NPA, epicarditis, and thrombosis that also contained Mac-1-positive-staining cells (Figure 4B).

### Table 6. Occurrence of Coronary Vasculitis in MRL/lpr Mice

<table>
<thead>
<tr>
<th>Diet/sex (n)</th>
<th>Large coronary artery cross sections</th>
<th>Small/medium coronary artery cross sections</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF Male (7)</td>
<td>12/52 (23.1%)</td>
<td>66/227 (29.1%)</td>
</tr>
<tr>
<td>Female (7)</td>
<td>17/75 (22.7%)</td>
<td>79/282 (28.0%)</td>
</tr>
<tr>
<td>Total (14)</td>
<td>29/127 (22.5%)</td>
<td>145/509 (28.5%)</td>
</tr>
<tr>
<td>Chow Male (3)</td>
<td>2/23 (8.7%)</td>
<td>13/136 (9.6%)</td>
</tr>
<tr>
<td>Female (4)</td>
<td>1/28 (3.6%)</td>
<td>5/148 (3.4%)</td>
</tr>
<tr>
<td>Total (7)</td>
<td>3/51 (5.9%)</td>
<td>18/284 (6.3%)</td>
</tr>
</tbody>
</table>

*Mice were fed a high-fat, high-cholesterol (HF) diet for 10-15 weeks.
FIGURE 5. Serum amyloid A (SAA) proteins in cardiac tissues from MRL/lpr mice fed the atherogenic diet for 15 weeks. Panel A: A medium-sized coronary artery with necrotizing polyarteritis had some positive staining for SAA1 and SAA2 in the lumen and media, but infiltrated mononuclear cells had no staining. Original magnification, ×30. Panel B: A region of the myocardium showing cell proliferation exhibited positive staining for SAA1 and SAA2. Original magnification, ×100. Panel C: A region of the epicardium exhibiting cell proliferation with numerous Mac-1-positive cells. Original magnification, ×50. Panel D: SAA3-positive staining in capillaries in the myocardium. Original magnification, ×100. Panel E: SAA3-positive-staining cells in the aortic valve attachment. Original magnification, ×100. The inset shows SAA1- and SAA2-positive staining in the aortic valve leaflet (arrowhead). Original magnification, ×50. Panel F: SAA3-positive-staining cells in an area of cellular proliferation in the epicardium. The inset shows CD4-positive-staining cells in the same area. Original magnification, ×150. All slides for immunohistochemical studies were counterstained with hematoxylin.
ApoB is the major structural protein of VLDL and LDL. Immunoblotting and immunoprecipitation studies have demonstrated the specificity of rabbit anti-rat apo B polyclonal antibody, which cross-reacts well against mouse apoB-100 and apoB-48.26 Positive staining with this antibody was found in the walls of the same coronary arteries that stained positively with oil red O. The staining usually occurred in the media and intima of affected coronary arteries (Figure 4C).

The SAA proteins are a family of acute-phase reactants whose levels rise dramatically after acute injury and are persistently elevated in chronic inflammatory states such as chronic liver diseases, rheumatoid arthritis, and various lymphoproliferative disorders.46-50 The SAA1 and SAA2 proteins are produced primarily in hepatocytes and, on sepsis, become associated with HDL. SAA3, on the other hand, appears to be produced primarily by macrophages.50 Using monospecific, polyclonal antisera to SAA1, SAA2, and SAA3 proteins, we observed intense positive staining in the intercellular space, capillaries, and cellular infiltrations in the myocardium of MRL/lpr mice fed the atherogenic diet (Figures 5B and 5D). Coronary arteries that had vasculitis showed some positive staining in the intima and in cellular infiltrates. Areas of necrotizing coronary arteritis involving mononuclear and neutrophilic cell infiltration had no antibody staining, except for some positive staining in the intima and media (Figure 5A). Macrophages, which were predominantly present in heart connective tissues, epicardium, and aortic valve leaflets, exhibited strong SAA3-positive staining (Figures 5E and 5F).

Discussion

MRL/lpr mice develop severe autoimmune disease and vasculitis by 5 months of age, whereas congenic MRL/n mice exhibit much milder vasculitis with a relatively late age of onset. We have studied these strains to examine interactions between vasculitis, hyperlipidemia, and arterial lipid accumulation. Hyperlipidemia was induced using a high-fat, high-cholesterol diet previously shown to result in high levels of atherogenic plasma lipoproteins in most strains of mice. Strain MRL/lpr mice maintained on the atherogenic diet exhibited dramatic increases in lipid accumulation in the coronary arteries and an increase in the incidence of myocardial infarction compared with MRL/lpr mice maintained on a chow diet or MRL/n mice on either diet. The atherogenic diet also accelerated autoimmunemediated vasculitis.

As in most other strains, MRL mice exhibited low levels of LDL and VLDL and high levels of HDL when maintained on a chow diet. When challenged with the high-fat, high-cholesterol, atherogenic diet, the levels of LDL and VLDL increased greatly, while HDL levels were not significantly affected. One striking difference between MRL/n and MRL/lpr mice was noted: the final levels of LDL and VLDL after 15 weeks on an atherogenic diet were significantly greater for MRL/n mice. Since the two strains are genetically very similar except for the lpr gene region, this suggests an interaction between the autoimmune disease and plasma lipoprotein metabolism. It is noteworthy that administration of certain cytokines, such as macrophage colony stimulating factor, to humans and experimental animals results in a dramatic lowering of LDL-C levels.51 Thus, it is possible that increased expression of such cytokines in MRL/lpr mice may account for the decreased levels of these atherogenic lipoproteins in plasma. The accumulation of apoB-containing lipoproteins at sites of vasculitis could also increase the turnover of these lipoproteins in plasma, thereby contributing to decreased plasma levels. Finally, it is possible that the differences in plasma lipoproteins between MRL/n and MRL/lpr mice result from an unknown gene distinct from lpr, differing between the strains. Such a gene could affect lipoprotein metabolism either directly or indirectly by its effects on inflammatory processes.

A number of significant interactions between hyperlipidemia and coronary vasculitis were observed. On a chow diet, little or no lipid accumulation occurred in the coronary arteries of either MRL/n or MRL/lpr mice, whereas on the atherogenic diet, lipid accumulation occurred in both strains (Table 5). We used a modification of the method of Paigen and coworkers52 to quantify lipid accumulation in coronary arteries. After 15 weeks on an atherogenic diet, MRL/lpr mice exhibited 10-20-fold greater lipid-containing lesions than MRL/n mice (p<0.01). On the other hand, after 15 weeks on the diet, the nonautoimmune strain C57BL/6J exhibited little or no lipid staining of coronary arteries. Thus, the degree of lipid accumulation was correlated with the degree of vasculitis. It is likely that the lipid accumulation was due in part to increased permeability of the artery wall resulting from endothelial cell damage. Consistent with this possibility was our finding that several plasma proteins, including apoB, SAA1, and SAA2, colocalized with lipid and vasculitis.

The high-fat, high-cholesterol diet also substantially increased the degree of vasculitis and myocardial infarction in MRL/lpr mice. Thus, on a chow diet ~6% of both large and small coronary arteries exhibited vasculitis, whereas on an atherogenic diet ~23% of large coronary arteries and ~29% of small and medium-sized coronary arteries exhibited vasculitis. The increased vasculitis was accompanied by increased myocardial infarction and mortality. On the basis of our pathological studies, it appears that the myocardial infarctions resulted from lesions of the proximal coronary arteries. The high incidence of myocardial infarction in MRL/lpr mice fed the atherogenic diet suggests that lipid accumulation secondary to vasculitis (both NPA and DVD) increased the occurrence of occlusion of the affected coronary arteries. Thrombus formation was frequently observed in DVD. This was presumably a consequence of platelet aggregation related to the presence of immune complexes in the circulation and artery wall and to endothelial damage.4 The mechanisms by which lipid accumulation may accelerate vasculitis are unclear, although recent studies suggest that lipids become oxidized in the artery wall and that certain lipid peroxides induce oxidative stress and activate various inflammatory genes, including monocyte chemotactic protein-1.12-15 Our immunohistochemical studies revealed that macrophages, neutrophils, and both T and B lymphocytes were present in coronary arteries exhibiting vasculitis and lipid accumulation. A previous study failed to find increased vasculitis in MRL mice maintained on a high-fat, high-cholesterol diet.50 A difference between that study and ours is that we used a more quantitative
method for examining lesion development. Moreover, our study was restricted to coronary arteries.

In contrast to coronary arteries, lipid accumulation in the aortic root was not enhanced in MRL/lpr compared with MRL/n mice. A wide range of susceptibility to aortic fatty streak development has been observed among different inbred strains of mice maintained on an atherogenic diet.\(^2^7,\!^2^8\) A comparison with some of these strains indicated that MRL/n mice are relatively susceptible, whereas MRL/lpr mice are relatively resistant. Since hyperlipidemia is a prerequisite for aortic lesion development, the decreased size of fatty streaks in MRL/lpr compared with MRL/n mice may be due to the lower levels of plasma LDL and VLDL in MRL/lpr mice.

Our results demonstrate that several members of the SAA family of proteins accumulate at the sites of vascular lesions and lipid accumulation. These include SAA1, SAA2, and SAA3. SAA1 and SAA2 are acute-phase reactants synthesized in the liver and transported in the circulation as complexes with HDL.\(^4^6-\!^4^9\) SAA3, on the other hand, appears to be expressed primarily by tissue macrophages.\(^5^0\) Our findings contrast with a previous study in which heart tissue of casein-treated mice failed to accumulate significant SAA proteins.\(^4^6\) The explanation for this may be due in part to the fact that casein treatment induces an acute inflammatory response, whereas autoimmune disease in MRL/lpr mice results in chronic inflammation. It is noteworthy that an atherogenic diet can induce the expression of SAA1 and SAA2 in certain strains of mice, apparently as a result of the accumulation of oxidized lipid.\(^5^2\) The SAA proteins are likely to affect both lipoprotein metabolism and inflammatory processes. Thus, SAA proteins influence the interactions of HDL with macrophages and other cells, oxidative bursts in neutrophils, tissue collagenase expression, and lymphocytic responses to antigens.\(^5^3\)

A number of studies have revealed that premature coronary atherosclerosis and myocardial infarction are observed in patients with SLE.\(^3^3-\!^3^6,\!^3^4\) However, it has been argued that this association may result from long-term treatment with steroids.\(^5^4\) SLE exhibits a number of similarities to the autoimmune disease of MRL mice; for example, both have high levels of circulating antibodies and arteritis and exhibit immune complexes in the vessel wall.\(^5^6\) Our studies, indicating that autoimmune vasculitis promotes lipid deposition in both large and small coronary arteries, are consistent with the hypothesis that inflammation of the vessel wall may be a promoting factor to the premature atherosclerosis observed in patients with SLE.\(^3^6\) Supporting this possibility is accumulating evidence from studies with nonhuman primates and humans that chronic, sustained, circulating immune complexes are likely to accelerate atherosclerosis, even in the absence of classic risk factors.\(^3^2,\!^3^7\) The lesions associated with circulating immune complexes represent a distinct form of concentric and transmural atherosclerosis and have been classified as "atheroarteritis." It is noteworthy that the lesions in MRL/lpr mice are also frequently of the concentric variety, with medial involvement.

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References

3. Murphy ED: Lymphoproliferation (lpr) and other single-locus models for murine lupus, in Gershwin ME, Merchant B (eds):


Immune-complex-mediated vasculitis increases coronary artery lipid accumulation in autoimmune-prone MRL mice.

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