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Original Articles

Coronary Arterial Lesions in Sexually Mature Non-Layers, Layers, and Roosters

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Summary. The effects of hereditary hyperlipidemia on coronary artery atherosclerosis were studied in 77 White Leghorn (DeKalb strain) chickens ranging from 4 to 13 months in age. After pubescence, the plasma levels of triglyceride and cholesterol in non-laying hens ranged 2- to 3-fold and 2- to 7-fold higher compared to layers. Serial sectioning revealed that most lesions were found in the proximal portions of both the left and right coronary arteries. Ultrastructurally, lesions in the roosters contained no foam cells, whereas some foam cells and small amounts of stainable lipid were observed in the thickened intima of layers. Half of the non-layers had stenotic lesions characterized by many foam cells, necrotic foci, and heavy stromal lipid deposits. Continuous permeation of excess plasma lipids into the arterial wall appeared to be an important factor in the development of coronary lesions.

Key words: Atherosclerosis – Chicken – Coronary artery – Hyperlipidemia.

The mutant White Leghorn "restricted ovulator" hen is characterized by a severe endogenous hyperlipidemia which results in severe aortic atherosclerotic lesions (Ho et al. 1974; Mitchell et al. 1979; Cho 1979; Tokuyasu et al. 1980). However, the extent of coronary atherosclerosis in these birds is questionable because: 1. the cell composition of chicken aorta and coronary artery are exactly different (Moss and Benditt 1970a); 2. chicken coronary arteries have been reported to be resistant to atherosclerosis (Moss and Benditt 1970b; Pick et al. 1953). We investigated age related changes in coronary arteries of restricted ovulator hens in order to clarify the issue of chicken coronary artery resistance to atherosclerosis, and to elucidate the process of endogenous coronary atherogenesis.

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Table 1. Age distribution of DeKalb, White Leghorn chickens

Sex	Age (months)				Total by sex
	4–5	6–7	8–9	12–13	
Rooster	6	6	7	6	25
Pullet	6	–	–	–	6
Layer	–	10	6	6	22
Non-Layer	–	8	7	9	24
Total	12	24	20	21	77

Materials and Methods

A total of seventy-seven 4 to 13 month old chickens were studied: 6 cockerels, 19 roosters, 6 pullets, 22 layers and 24 hereditary non-layers (Table 1). The chickens were kept at an 18 h per day photoperiod, housed in individual cages, and fed a commercial mash ad libitum throughout the experiment. The chicken mash contained 2% crude fat and a trace amount of cholesterol. Non-layers in this study laid no eggs at all. Blood samples were taken by cardiac puncture using a heparinized syringe. Plasma total cholesterol concentrations were enzymatically determined using a commercial cholesterol reagent (Abbott Laboratories, Chicago, Illinois). Triglyceride concentrations were determined by the Foster and Dunn method (1973).

Chicken coronaries were preserved by immediately perfusing cold 1.4% glutaraldehyde in phosphate buffer (pH 7.4) through the heart. The proximal 4 mm extramural segments of both the left and right coronary arteries were dissected, and tissue blocks were further fixed in 1% phosphate-buffered osmium tetroxide (pH 7.4). After alcohol dehydration, blocks were embedded in epoxy resin (Epon 812). Thin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi HU-12 electron microscope.

One micron thick sections for light microscopy were cut from the middle of blocks and stained with alkaline toluidine blue. These thick sections were used for histological examination of lesions and also for histometric evaluation of the degree of intimal thickening. The degree of the intimal thickening was measured by a cut-and-weigh method (Toda et al. 1978; Toda et al. 1980). Photomicrographs of each section were projected; the degree of intimal thickening was expressed by the ratio of the surface area of the intima relative to the surface area circumscribed by the internal elastic lamina.

For quantitative comparisons of degenerated smooth muscle cells in the coronary arterial wall, more than 150 cells with a nucleus were counted on both left and right coronary arteries of each bird at a constant magnification of 5,000. For the determination of coronary artery lesion distribution, two transverse slices were obtained from the hearts of three 13 month old non-layers, embedded in parafin, and then seven micron serial sections were stained with hematoxylin eosin and Masson's trichromeelastica Van Gieson.

Results

I. General Pathological Findings

Table 2 lists the plasma triglyceride and cholesterol levels in roosters, laying hens, and non-laying hens. The levels of plasma triglyceride and cholesterol in hens was higher than those in roosters at pubescence (4 to 5 months). After the age of 6 months, the plasma levels of triglyceride and cholesterol in non-layers ranged 2- to 3-fold and 2- to 7-fold higher compared to layers. The plasma lipid levels declined with advancing age in both layers and non-layers.

Table 2. Plasma triglyceride and cholesterol levels in chickens of varying age

Sex	Age (months)			
	4–5	6–7	8–9	12–13
Triglyceride (mg/dl)				
Rooster	106.2 ± 9.6	122.1 ± 13.5	148.0 ± 19.7	129.0 ± 18.5
Pullet	1,756.5 ± 103.3	–	–	–
Layer	–	3,488 ± 206.3	2,824.8 ± 296.5	1,115.3 ± 85.0
Non-layer	–	6,893.3 ± 521.5	7,294.3 ± 694.7	4,123 ± 325.2
Total cholesterol (mg/dl)				
Rooster	97.5 ± 7.9	156.0 ± 16.7	86.3 ± 13.8	110 ± 15.5
Pullet	212.0 ± 95.7	–	–	–
Layer	–	247.5 ± 68.0	235.0 ± 43.2	225.0 ± 36.6
Non-layer	–	638.4 ± 77.9	719.8 ± 105.6	481.1 ± 60.9

mean ± standard error

Nine of 77 birds examined had myocardial collections of macrophages and lymphocytes. In these 9 cases, the adventitia and media of intramural coronary arteries and veins had occasional chronic inflammatory cellular infiltrates which appeared to be involved secondarily to myocarditis. Most of these cases did not have severe proximal extramural coronary atherosclerotic lesions, and inflammations occurred in both left and right ventricles, implying little relationship between the myocarditis and the atherosclerotic process. Rather, these cases of myocarditis appeared related to exogenous infection common to the avian species. Moderate myocardial fibrosis was observed in the anterior region of the left ventricle of only one bird, which was a non-layer with severe atheromatous lesions in the left coronary artery. This fibrotic lesion appeared compatible with ischaemic heart disease.

Serial sections of both intramural and extramural coronary arteries revealed that lesions present in the intramural coronaries were mild and non-occlusive, whereas the lesions of both the left and right proximal extramural coronaries were severe; therefore, major attention was given to the extramural regions. Figure 1 illustrates the incidence per chicken, and degree of stenosis of the extramural coronary arteries. Roosters had a maximum of 10% stenosis after the age of 6 months; this luminal narrowing gradually increased with age. The degree of stenosis was more severe in non-layers than in layers and roosters. Non-layers showed severe atherosclerotic stenotic lesions even at 6 months of age. The most severe stenosis was observed in the 13 month old group.

II. Ultrastructural Findings

Coronary Arterial Lesions in Roosters. In most of the coronary arteries of roosters, a layer of endothelial cells closely covered the internal elastic lamina with occasional fenestration. Mild irregular intimal fibromuscular thickening was

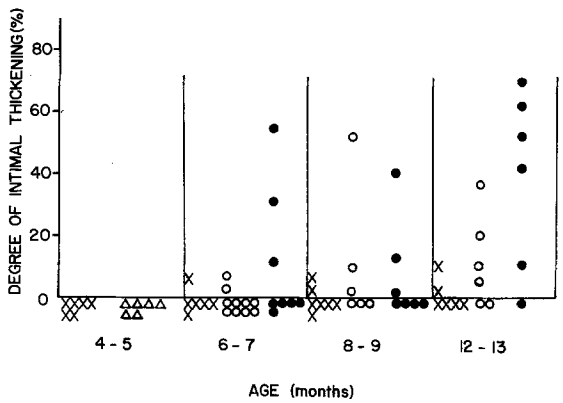

Fig. 1. Degree of intimal thickening in cockerels/roosters (×), pullets (△), layer hens (*open circle*), and non-layer hens (*closed circle*). Each symbol below the zero line represents a chicken with no intimal thickening. Symbols above zero line show degree of intimal thickening for each bird. At 12–13 months old, non-layers had a significantly greater degree of intimal thickening than layers or roosters ($P < 0.05$, *t*-test)

observed in older roosters. The media consisted of 8 to 9 layers of smooth muscle cells; no foam cells were present in either the intima or the media of coronary arteries. Endothelial cells had junctions with 100 to 200 Å spaces. Endothelial cytoplasm contained few mitochondria, scant endoplasmic reticulum, and occasional fine filaments. Thickened intima consisted of several layers of smooth muscle cells (Fig. 2). Occasional poorly differentiated cells were observed in the subendothelial space. The stroma of thickened intima contained glycosaminoglycans, collagen fibers, and dense particles. A few inner medial smooth muscle cells extended cytoplasmic processes to the subendothelial space through fenestrae in the internal elastic lamina. Modified smooth muscle cells were scattered in the inner media, expecially near the thickened intima. Small amounts of cell debris were seen in the thickened intima and media of older roosters.

Coronary Arterial Lesions in Layers. The endothelial cells in normal areas contained more vesicles in layers than roosters, and the subendothelial stroma had more dense particles. Foci of foam cells were scattered in the thickened intima. In these foci, the endothelial cells were flat and the subendothelial basement membranes were focally thick. The subendothelial spaces contained many irregularly arranged smooth muscle cells. Occasional cholesterol clefts were present in some of these intimal smooth muscle cells. The internal elastic lamina appeared thin, but had no overt disruption or duplication. Modified smooth muscle cells in the inner media were more numerous in layers than in roosters. Most medial smooth muscle cells with well developed myofilaments and fusiform densities had a few lipid droplets; medial stroma contained increased glycosaminoglycans, collagen fibers, and cell debris compared to roosters. In a few 13 month old layers, deposition of extracellular dense lipid granules was extensive in deep intima and inner media (Fig. 3).

Coronary Arterial Lesions in Non-Layers. The presence of many foam cells characterized the thickened intima and the media in non-layers with or without overlying stenotic lesions. This intimal and medial foam cell accumulation was

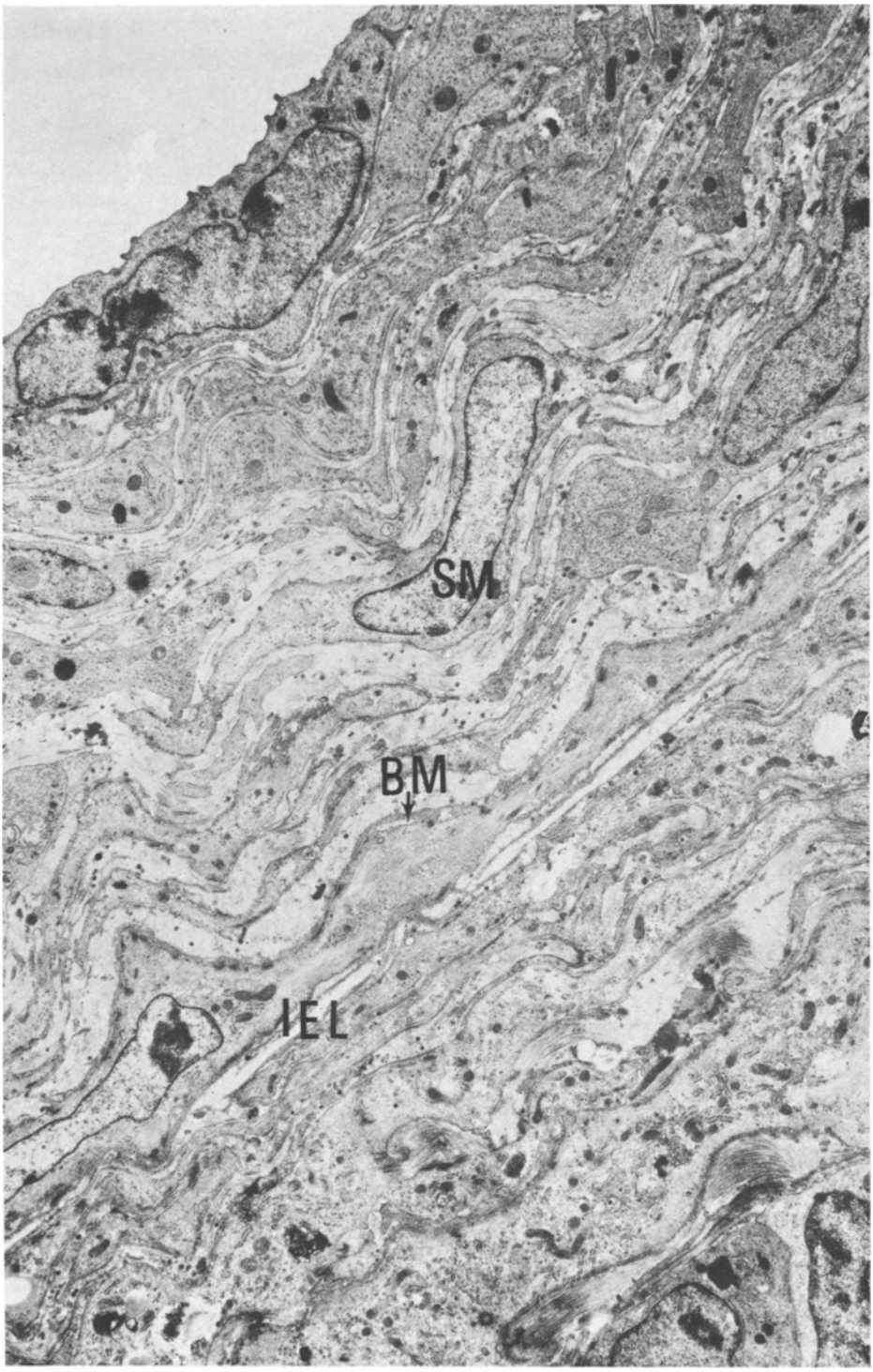

Fig. 2. Thickened intima of a coronary artery in a 13 month old rooster. (×6,000). *BM:* basement membrane. *IEL:* internal elastic lamina. *SM:* smooth muscle cell

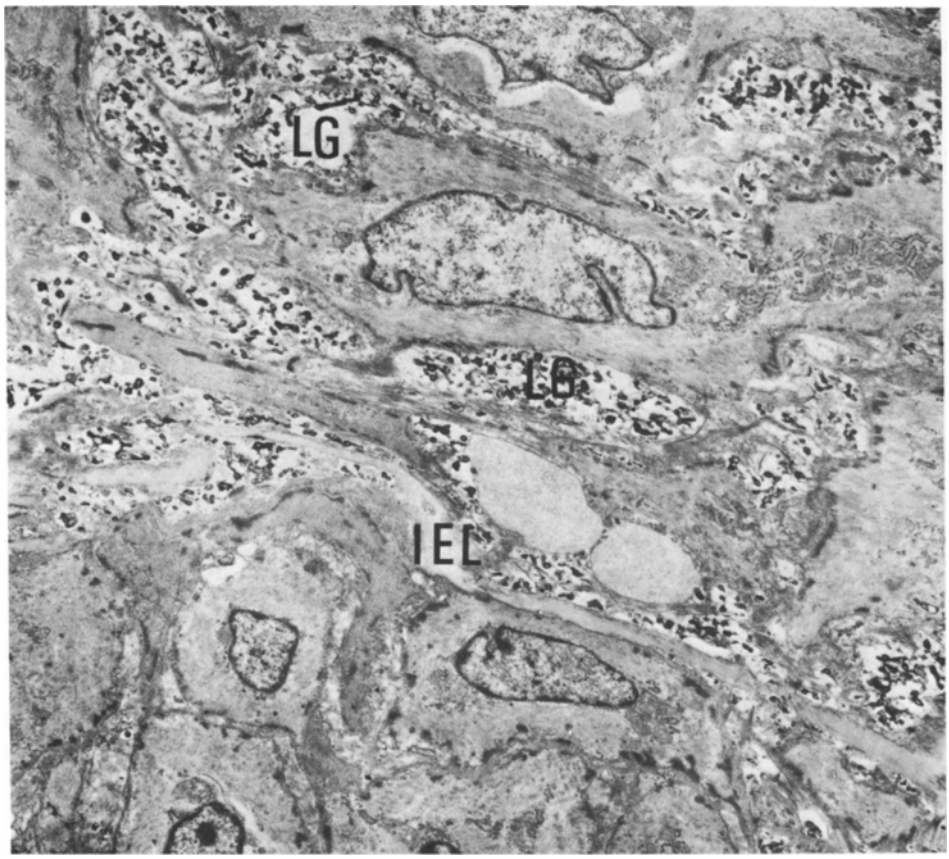

Fig. 3. Laying hen, 13 months of age. Extracellular lipid granules *(LG)* are more heavily deposited in the intima *(upper portion of the micrograph)* than in the media. (×6,000). *IEL:* internal elastic lamina

observed in two out of eight 6 to 7 month old, three out of seven 8 to 9 month old, and four out of nine 12 to 13 month old non-layers. No thrombus or capillary formation was present in thick atheromatous lesions. The internal elastic lamina was discontinuous in severe lesions, and occasional cholesterol clefts were observed in the intimal cells. Cell types present in lipid-rich intimal lesions were: foam cells, smooth muscle cells, poorly differentiated cells (Fig. 4), and plasma cells. Lipid droplets and lysosomal dense bodies were seen in endothelial cells (Fig. 4).

Two types of subendothelial foam cells were distinguishable: one was confined to the subendothelial area and had no basement membrane or other features of smooth muscle cells (Fig. 5a). The other was deeper in the intimal lesions and had a basement membrane (Fig. 5b). Extracellular dense lipid granules were heavily deposited in the deep intima and inner media. Lipid droplets of various size and structure were observed in medial smooth muscle cells (Fig. 6). Necrotic cells were observed more frequently in the intima and inner

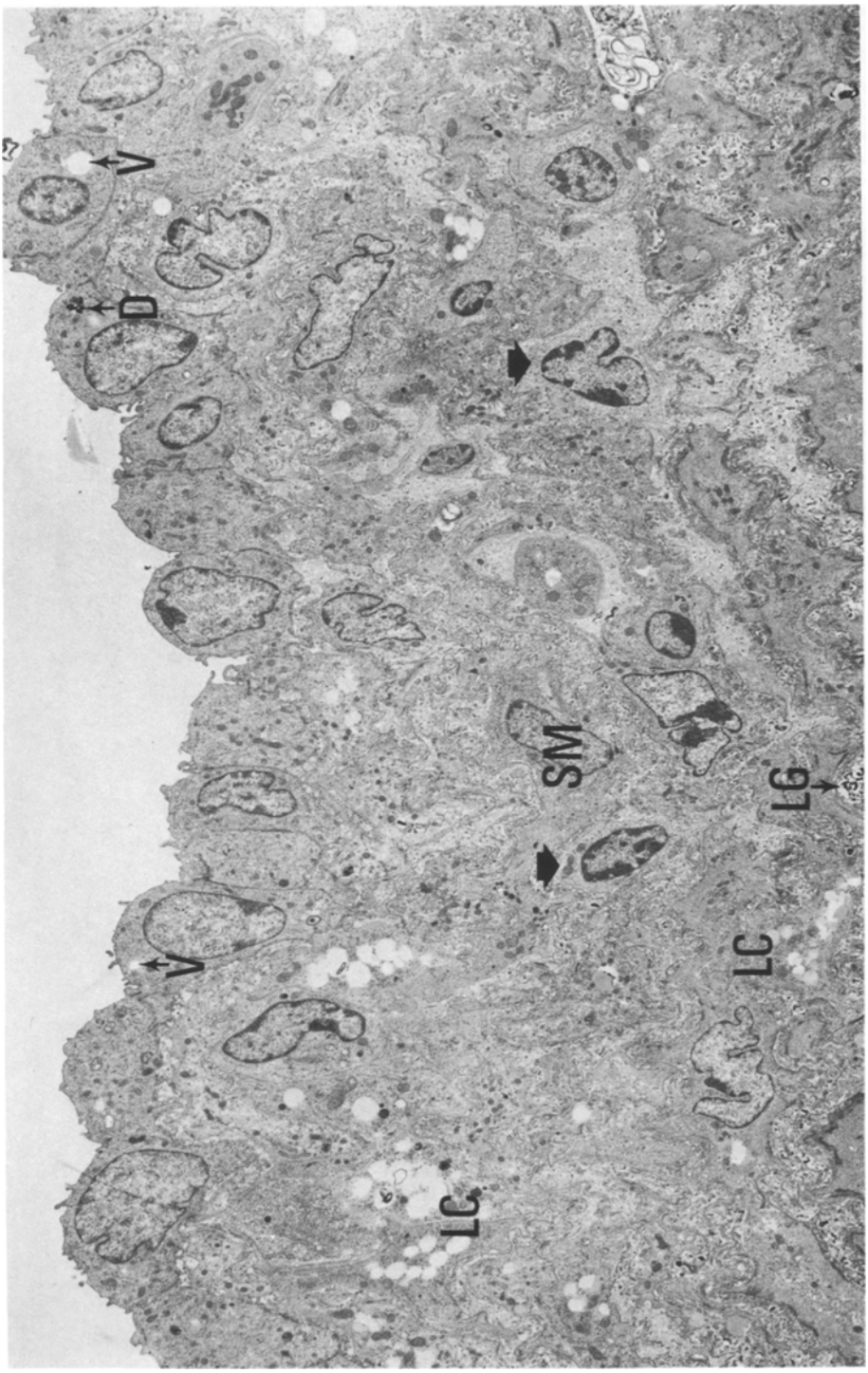

Fig. 4. Thickened coronary intima of 6 month old non-laying hen. The endothelial cells contain lipid droplets (*V, arrow*) and dense lysosomal material (*D, arrow*). (×3,000). *LC:* lipid-containing cells. *LG:* extracellular dense lipid granules. *SM:* smooth muscle cell. Note poorly differentiated cells (*big arrows*)

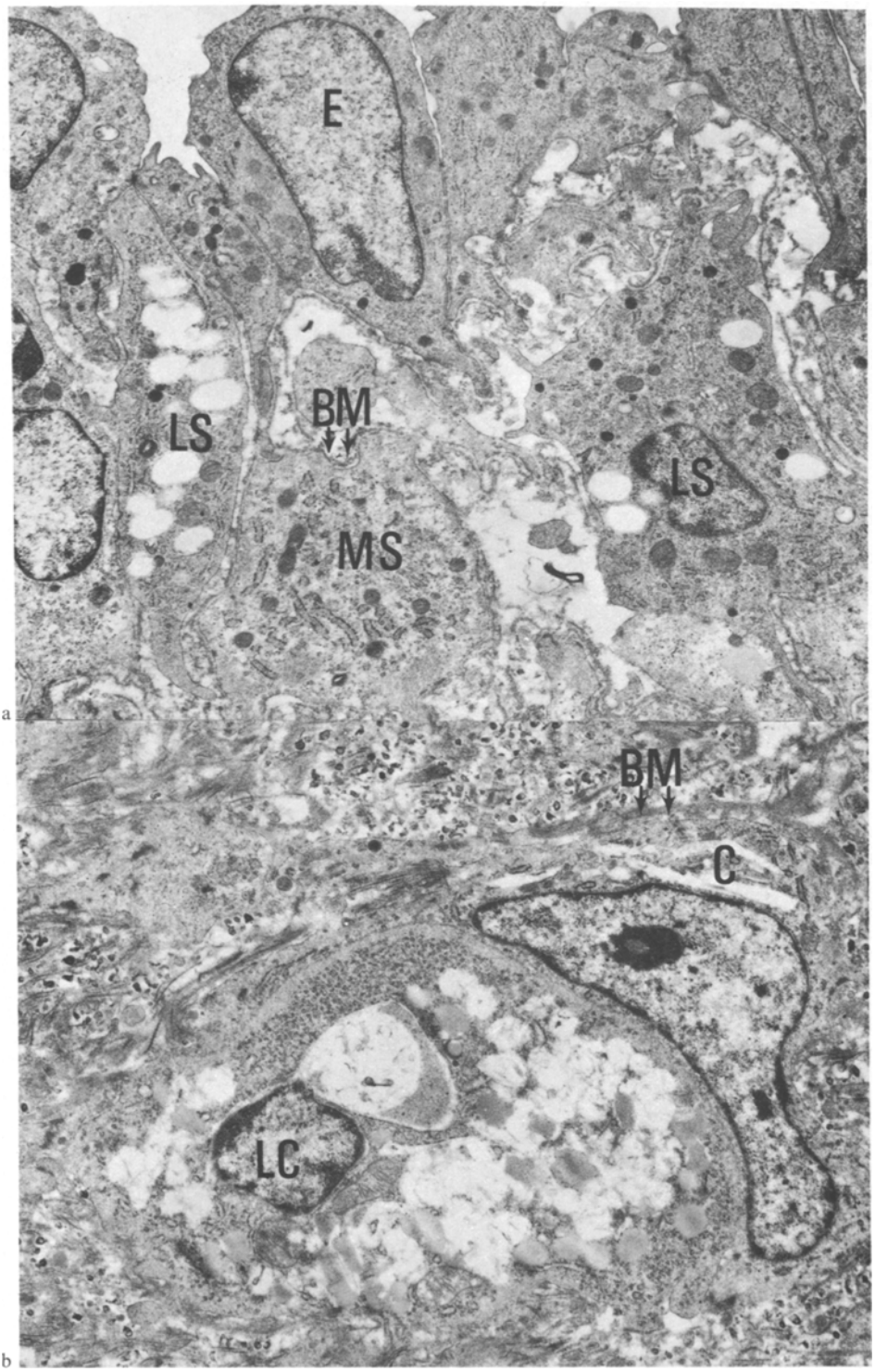

Fig. 5. a Foam cells in the thickened intima of a non-laying hen. Lipid-containing spindle to stellate cells (*LS*) are present beneath the endothelial cells (*E*). Modified smooth muscle cells (*MS*) are surrounded by basement membrane (*BM, arrow*). (×9,600). **b** Lipid containing smooth muscle cells (*LC*) with obscure cytoplasmic outlines in deep intima. (×7,200). *BM:* basement membrane. *C:* cytoplasmic cholesterol clefts

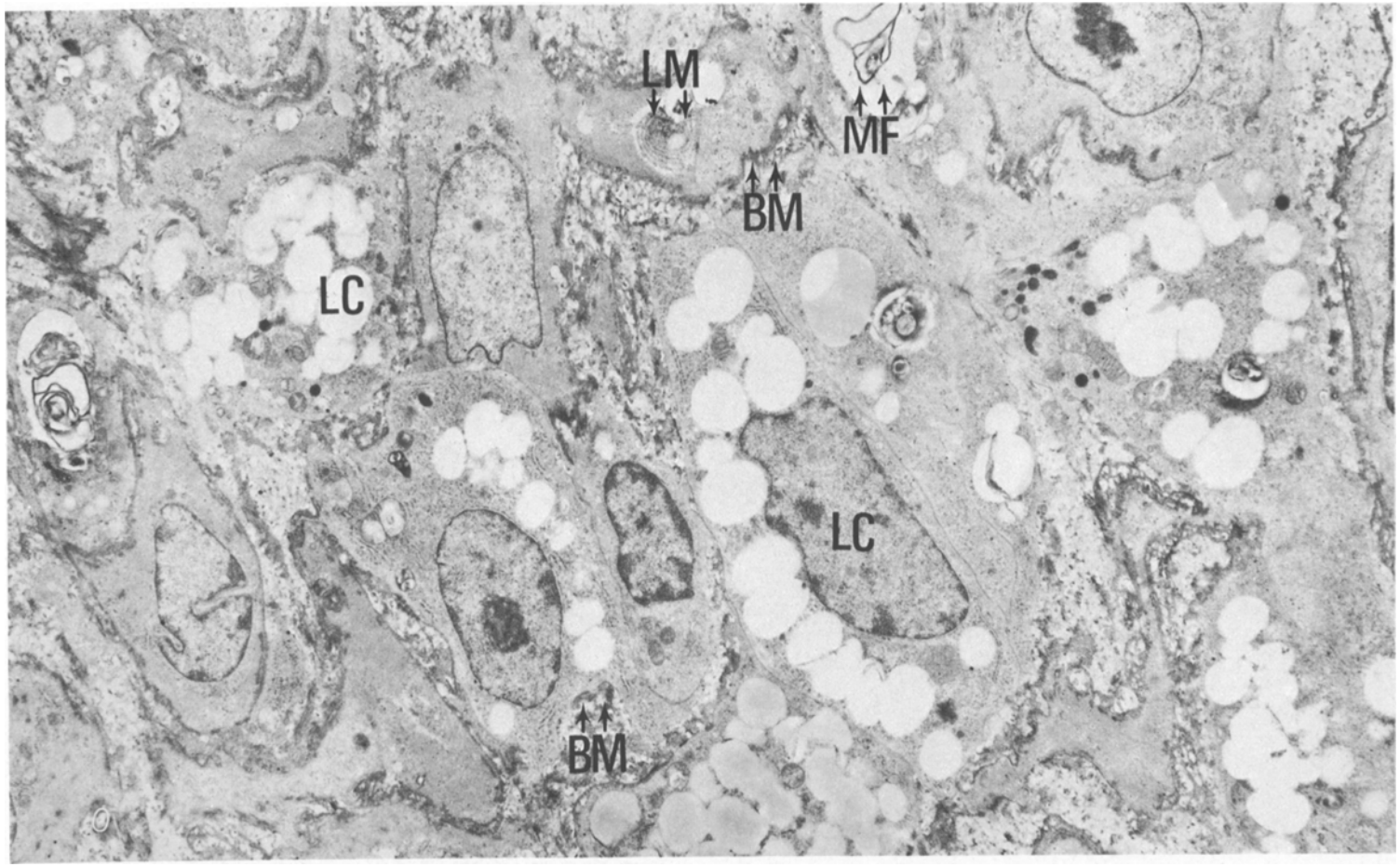

Fig. 6. Medial layer of coronary artery from a 6 month old non-laying hen. Many lipid containing smooth muscle cells (*LC*) with thick basement membrane (*BM, arrow*) are apparent; some of these cells also contain laminated material (*LM, arrow*), and myelin figures (*MF, arrow*). (×6,000)

Table 3. Frequency and percentage of degenerated smooth muscle cells

Group	Type of degenerated cell		Combined
	Rarefaction	Pyknosis	Total cell count
(f)	36	6	42
Rooster			3287
(%)	(1.1)	(0.2)	(1.3)
(f)	76	29[a]	105
Layer			3604
(%)	(2.1)	(0.8)	(2.9)
(f)	175	58[a]	233
Nonlayer			4855
(%)	(3.6)	(1.2)	(4.8)

[a] Except for pyknosis between layers and non-layers, all other comparisons among the three groups are significantly different by Chi Square statistics ($P < 0.05$)

media of coronary arteries from non-layers compared to layers. Severe lesions in 13 month old non-layers contained accumulations of foam cells, and extracellular dense lipid granules were observed throughout the entire artery wall. Cholesterol clefts were seen in the outer media and adventitia. Occasional mitotic smooth muscle cells were seen in inner media.

The majority of dead cells were characterized by swollen and rarefied cytoplasm (cytolysis). Severely damaged cells had marked nuclear pyknosis and no definite cytoplasmic boundary. Counts and computations of degenerated smooth muscle cells from both coronaries of 6 to 13 month old chickens are shown in Table 3. Cytolysis type degeneration was more frequent in non-layers than in layers or roosters. Pyknotic type degeneration was low in all groups, but was significantly higher in non-layers compared to layers or roosters.

Discussion

Early observations of spontaneous and experimental forms of chicken coronary atherosclerosis consistently indicated that the disease originated in the intima (Dauber and Katz 1942). Later Paterson et al. (1948) and Lindsay and Chaikoff (1950) questioned this hypothesis because they found that in some cases, spontaneous coronary atherosclerosis had medial origins. Paterson (1965) later explained the etiology of the medial lesions as being due to infectous lymphomatosis, a disease which is common in chickens under one year of age but which tends to disappear in older birds. Recent chicken experiments also suggest that viral diseases such as Marek's disease also result in myocardial and coronary arterial lymphocytic infiltration (Fabricant et al. 1978; Minick et al. 1979). In our study, focal collections of lymphocytic cells were observed in the epicardium or myocar-

dium in 9 of 77 chickens less than one year old. These mild myocardial lesions secondarily involved coronary arterioles and venules, and appeared to be unrelated to hyperlipidemia induced atherosclerosis. Therefore, the myocarditis observed in our study appeared compatible with the observations of Paterson (1965) who described viral related lymphomatosis as being involved in certain forms of chicken coronary sclerosis. In only one bird, moderate myocardial fibrosis was observed in the anterior region of the left ventricle; this bird was a 13 month old non-layer with severe atheromatous lesions in the left coronary artery. This fibrotic lesion appeared compatible with ischaemic heart disease as described by Doerr et al. (1974) in older hens where severe atheromatous coronary lesions were found to be related with myocardial "elektive Parenchymnekrose".

The focus of our study involved the atherosclerotic lesions in the extramural coronary arteries, which were distinct from and much more severe than the inflammatory lesions described above. The thickened intima from extramural segments of non-layer coronary arteries contained many foam cells, while roosters had no foam cells. Foam cells, chracteristic of atherosclerotic lesions, have been reported to originate from both macrophages and smooth muscle cells (Marshall and O'Neal 1966). In the present study, foam cells originating from smooth muscle cells clearly played the key role in the development of the severe extramural coronary atherosclerotic lesions; later, hematogenous cells participated in the development of more advanced lesions. The incidence and severity of extramural coronary intimal thickening and stenosis in roosters, laying hens, and non-laying hens was most evident at the age of 12 to 13 months. Intimal cells in rooster lesions consisted of mature and modified smooth muscle cells and poorly differentiated cells. The thickened intima of laying hens had occasional small clusters of lipid-containing smooth muscle cells. The severe atheromatous lesions observed in non-laying hens contained not only foam cells, but also necrotic and mitotic smooth muscle cells. Various factors such as aging (Joris and Majno 1974), hemodynamic stress (Stehbens 1975), and atherogenic diet (Imai et al. 1970), cause an increase of cell debris in arterial wall. The frequent presence of cell necrosis in non-laying hens reemphasizes that smooth muscle cell necrosis is one of the major anatomic features in the development of atherosclerosis.

In our study, the severity and speed of coronary atherosclerosis development appeared to be highly correlated with severity of plasma hyperlipidemia. Since the chicken diet contained only a trace of cholesterol, the excess plasma cholesterol in layers and hereditary non-layers was produced endogenously, due to increased liver synthesis (Mitchell et al. 1979). This point is important in view of recent evidence that purified crystalline cholesterol, used in dietary supplements, contains varying amounts of contaminating oxidized cholesterol derivatives which are highly angiotoxic (Imai et al. 1976; Taylor et al. 1979). Dietary cholesterol supplementation causes hyperlipidemia in chickens, but the pathology and plasma lipid profiles are different. Moss and Benditt (1970b) reported that 2 months cholesterol feeding in cockerels resulted in severe aortic lesions, but the coronaries were unaffected. In chickens and other animals, cholesterol feeding usually results in a dramatic increase in serum cholesterol but little

change in serum triglyceride. In our study, non-layers as well as layer hens were hypertriglyceridemic as well as hypercholesterolemic; this is also a common feature in human familial hyperlipidemia.

Plasma estrogen and estrone levels have been reported to be 2–3 fold higher in non-layer hens compared to layers (Schjeide et al. 1976); estrogen levels in layers are higher than in pullets or roosters. We have recently observed in our laboratory that 2–8 weeks feeding of 17β-estradiol as a 0.05% dietary supplement to week old chicks results in plasma lipid patterns similar to those seen in non-layers (manuscript in preparation).

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