Lipid Accumulation and Ultrastructural Change Within the Aortic Wall During Early Spontaneous Atherogenesis

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To identify the initial period and type of lipid accumulation during spontaneous atherosclerosis, quantitative chromatographic profiles of major lipid classes in upper thoracic aortas (non-lesion areas) and celiac artery cushions (lesion areas) were obtained from atherosclerosis-susceptible White Carneau (WC) and atherosclerosis-resistant Show Racer (SR) pigeons from 1 day to 6 months of age. Thoracic aortas of WC and SR pigeons contained similar amounts of cholesterol, nonesterified fatty acids, triacylglycerols, cholesteryl esters, phospholipids, and hydrocarbon at each age studied. However, celiac sites in WCs contained more total lipid than corresponding SR sites at 6 weeks and 6 months of age. This initial increase at 6 weeks in WCs was characterized by increased concentrations of nonesterified saturated fatty acids. By 6 months of age, WC celiac cushions had greater concentrations of each lipid class except hydrocarbon than did SR cushions. This initial lipid accumulation was accompanied by ultrastructural changes within the arterial wall, which included the presence of extracellular, vesiclelike structures and extensive accumulation of basal lamina-like material between cells. This material was not present in aortic regions that are not predisposed to lesion formation. This material increased by 6 months of age in the enlarging WC fibromuscular intimal cushions. These morphologic changes paralleled the quantitative lipid increases and represented the first morphologic changes detectable at this site. Age-related changes in arterial lipid content and ultrastructure in SRs are different from those related to early spontaneous atherogenesis in WCs. (Am J Pathol 1980, 100:683-706)

SINCE EARLY ATHEROSCLEROTIC LESIONS may be reversible or preventable,[1] it is important to understand their pathogenesis and to define initiating factors. In man, intimal thickening is found in the aorta,[2] and at bifurcations of other large arteries,[3] "pads" or "cushions" occur at specific anatomic locations rather than as diffuse thickening.[4] These intimal pads or cushions are considered the sites for lipid accumulation and subsequent atherosclerotic lesions.[5] A similar distribution of cushions has been observed in several other animal species, including the pigeon.[6-8]

Atherosclerosis-susceptible White Carneau (WC) and atherosclerosis-resistant Show Racer (SR) pigeons provide a unique system for the study of atherogenesis, since the pathogenesis in WCs is similar to that in man,[8-11] and the atheromatous lesions in the WC are highly predictable in terms of site specificity at the celiac artery bifurcation and the rate of pathogenic progression as a function of age.[8,12,13] Moreover, the SR serves as a useful control in distinguishing physiologic events associated with development from those characteristic of early atherogenesis in the WC.

Previous studies by Clarkson et al [9] and Santerre et al [8] revealed that paired muscular intimal thickenings occur at the celiac artery bifurcation in embryonic WCs with subsequent focal lipid accumulation by 6 months of age.[14] Lipid accumulation in developing fibromuscular intimal cushions in WCs could be attributed to endothelial injury,[15] altered energy metabolism,[16-18] accumulation of proteoglycans,[19] or focal hypoxia.[20] Whatever mechanism is responsible, cholesteryl esters become the predominant lipid as the lesion progresses beyond 6 months of development.[21]

Various morphologic studies of lesion development in the WC have been reported,[8,11,15,22,23] but few have directly correlated ultrastructural change within the vessel wall with lipid profiles at lesion sites during the early stages of the arteriopathy. Since it is not known when the initial lipid accumulation occurs in WC lesion sites, it is necessary to examine the lipid class composition at pre-lesion sites in aortas of younger pigeons (less than 6 months of age) as it relates to structure of the artery wall in order to gain insight into the mechanism(s) of intimal lipid accumulation, the key feature of atherosclerosis.

Although much is known regarding changes in aortic structure and lipid composition during "cholesterol-induced" atherosclerosis in pigeons,[24,25] there is little information available correlating sequential alterations in chemistry and morphologic characteristics within the arterial wall during early spontaneous atherogenesis. This article presents lipid-class profiles from thoracic and celiac foci of WC and SR pigeons during early spontaneous atherogenesis and correlates lipid differences between the two breeds with ultrastructural changes within the artery wall.

Materials and Methods

White Carneau and Show Racer pigeons 1 day, 6 weeks, 12 weeks, and 6 months old were used in this study. Eggs obtained from our colonies were incubated to provide the source of 1-day-old birds. Six-week-, 12-week-, and 6-month-old pigeons were purchased from Palmetto Pigeon Plant, Sumter, South Carolina. Pigeons from both sources were derived from the inbred lines described by Clarkson et al.[9]

Lipid Class Analysis

After the pigeons were exsanguinated, upper thoracic aortas and muscular foci at the celiac artery bifurcation were excised, placed in ice-cold Hanks' balanced salt solution (without glucose), and dissected free of adherent blood and perivascular tissue. Four pools of aortic tissue for each age–site combination within each breed were analyzed for lipid. Each pool contained segments of aortic tissue from birds of both sexes to provide at least 10–15 mg wet tissue weight (approximately 2 birds per pool).

Following homogenization of the aortic tissue in 1.0 ml ice-cold physiologic saline (pH 7.0), 0.30-ml samples were taken for DNA analysis by the microfluorometric method of Kissane and Robins,[26] with the use of 3,5-diaminobenzoic acid, modified to accommodate sample sizes of 0.1–1.0 μg DNA and for use with a Turner filter fluorometer. Prior to analysis, tissue samples for DNA determination were treated with 0.1 ml 0.9% Pronase (Calbiochem; nuclease-free) in 0.20 M Tris-HCl buffer (pH 7.8) for 12 hours to release all cellular DNA. Lipids were extracted from the remaining sample by the method of Folch et al.[27] Organic solvents used in these analyses were reagent grade and distilled prior to use. Exhaustive hydrogenation of the extracted lipid for 5 hours with platinum oxide as catalyst was necessary in order to quantitate the lipids fluorometrically.[28] After hydrogenation the lipid extract was filtered, evaporated to dryness, and dissolved in 10.0 ml methylene chloride: methanol (2:1, v/v). A 3.0-ml sample was subsequently fractionated by thin-layer chromatography on silica gel 60 plates (EM Laboratories, Elmsford, NY) [29] into six lipid classes: phospholipids, cholesterol, nonesterified fatty acids, triacylglycerols, cholesteryl esters, and hydrocarbons. All lipid classes except phospholipids were quantitated fluorometrically in situ.[28] Phospholipids were eluted and quantitated colorimetrically with 0.02% malachite green according to the method of Chalvardjian and Rudnick [30] in a Beckman Model 26 double-beam spectrophotometer. Lipid standards used have been described previously.[29]

Nonesterified Fatty Acid Analysis

Following homogenization of aortic tissue in 1.0 ml ice-cold physiologic saline (pH 7.0), samples for lipid and DNA analysis were taken as described in the previous section. Once the lipid was extracted and dissolved in 10.0 ml methylene chloride: methanol (2:1, v/v), a 4.0-ml sample was separated into lipid classes by thin-layer chromatography.[29] (This sample was not hydrogenated.) Nonesterified fatty acids were subsequently eluted from the silica gel plate by the method of Goldbrick and Hirsch.[31] Methyl esters were prepared by direct micromethanolysis with the use of boron trichloride gas,[32] and analyses were performed according to established gas–liquid chromatographic procedures.[32,33] The column was calibrated with Applied Science (State College, Pa) fatty acid standard mixture K-108, and agreed with the stated composition data with a relative error less than 4% for major components. Standard fatty acid methyl esters showed a linear response over the range of sample sizes analyzed, and gas chromatographic peaks were identified by the comparison of retention times with those of reference standards. Areas of chromatographic peaks were determined by triangulation.

Ultrastructure

Twenty-six WC and SR pigeons at 6 weeks and 6 months of age were used in this portion of the study and were taken from the same groups of animals used for the lipid determinations. The animals were anesthetized with Nembutal, 4 mg/100 g body weight (Sodium Pentobarbital, Abbott Laboratories, North Chicago, Ill), injected through the axillary vein. The body cavity was immediately opened to expose the heart, and an 18-guage needle connected to a perfusion device was inserted into the left ventricle of the

heart. Perfusion was started initially with physiologic saline for 6 minutes and continued with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 20 minutes at 40 C under a pressure of 140 mm Hg. An exit for the perfusion fluid was provided by cutting of the brachial and femoral arteries. After perfusion, the entire aorta was dissected free, and the thoracic and celiac regions were cut into 1-mm rings and immersed in the glutaraldehyde fixative (as above) for an additional 2 hours at room temperature. The tissue was rinsed overnight in 0.1 M cacodylate buffer, stained *en bloc* with uranyl acetate, dehydrated in graded alcohols, and embedded in Epon 812.[34] Thin sections were cut with a diamond knife, stained with saturated uranyl acetate and lead citrate,[35] and examined in a Philips EM 200 electron microscope.

Analysis of Lipid Data

Lipid data obtained from the muscular cushions at the celiac axis and from upper thoracic aortas of WC and SR pigeons were compared statistically with analyses of variance.[36]

Results

Quantitative Lipid Analysis

Total Lipid Composition

There were no significant sex-related differences between the amounts of any lipid class in WC or SR pigeon aortas at either site in any of the four age groups studied. Therefore, values for both sexes were grouped together for presentation in the tables and graphs (an equal number of males and females were used in each case).

Age Trends and Breed Differences: Celiac foci in WC had significantly more total lipid at 6 weeks and 6 months of age than SR celiac foci, the largest difference being at 6 months of age (Table 1). Moreover, total lipid in WC and SR celiac foci increased significantly ($P < 0.05$) at each successive age studied after 1 day. A similar increasing trend in total lipid was observed in thoracic aortas of both breeds, but the amount of increase was much less, and there were no major differences between breeds in total lipid or in any individual lipid class (Tables 3 and 4).

Although cholesterol was a predominant lipid in both WC and SR celiac foci at 1 day and showed a marked increase by 6 weeks, it was only at 6 months of age that there was a major difference between WC and SR celiac foci, WCs having significantly more than SR (Tables 1 and 2). By this age, however, cholesterol constituted a much lesser proportion of the total lipid in each breed. Nonesterified fatty acids (NEFA), the other major lipid class in 1-day WC foci, was greater than in SR foci and underwent a dramatic increase by 6 weeks, to plateau and remain significantly greater than SR celiac NEFA, despite an increase in the latter at each succeeding age (Tables 1 and 2).

Table 1—Lipid Content of Celiac Foci as a Function of Age in White Carneau (WC) and Show Racer (SR) Pigeons

	Cholesterol	Nonesterified fatty acids	Triacylglycerols	Cholesteryl esters	Phospholipids	Hydrocarbon	Total lipid
1 day							
WC	0.32 ± 0.05* (20.4)†	0.21 ± 0.03 (19.3)	0.04 ± 0.01 (3.7)	0.16 ± 0.02 (14.7)	0.13 ± 0.01 (11.9)	0.23 ± 0.04 (21.1)	1.0 ± 0.1
SR	0.34 ± 0.05 (30.0)	0.05 ± 0.02 (4.5)	0.06 ± 0.01 (5.5)	0.16 ± 0.01 (14.5)	0.28 ± 0.01 (25.5)	0.21 ± 0.04 (19.1)	1.1 ± 0.1
6 weeks							
WC	3.38 ± 0.87 (20.9)	5.56 ± 0.44 (34.3)	2.33 ± 0.43 (14.4)	0.31 ± 0.05 (1.9)	1.96 ± 0.35 (12.1)	2.65 ± 0.36 (16.4)	16.2 ± 0.8
SR	2.04 ± 0.28 (23.2)	0.63 ± 0.14 (7.2)	0.99 ± 0.01 (11.2)	0.44 ± 0.09 (5.0)	2.06 ± 0.31 (23.4)	2.65 ± 0.28 (30.0)	8.8 ± 0.4
12 weeks							
WC	2.53 ± 0.25 (11.2)	4.59 ± 0.83 (20.4)	4.14 ± 0.74 (18.4)	1.97 ± 0.17 (8.8)	3.49 ± 0.70 (15.5)	5.79 ± 0.42 (25.7)	22.5 ± 1.5
SR	1.03 ± 0.19 (7.0)	1.26 ± 0.23 (8.5)	2.54 ± 0.61 (17.2)	2.00 ± 0.37 (13.5)	3.89 ± 0.63 (26.3)	4.07 ± 0.16 (27.5)	14.8 ± 2.3
6 months							
WC	7.49 ± 0.59 (11.8)	6.01 ± 0.40 (9.5)	7.82 ± 1.25 (12.4)	6.91 ± 0.47 (10.9)	14.50 ± 2.40 (22.9)	20.54 ± 3.19 (32.5)	63.3 ± 4.4
SR	1.57 ± 0.08 (4.6)	3.13 ± 0.55 (9.3)	2.03 ± 0.05 (6.0)	1.44 ± 0.30 (4.3)	7.75 ± 1.37 (22.9)	17.89 ± 3.87 (52.9)	34.8 ± 4.0

* Values expressed as µg lipid/µg DNA ± SEM. Each value represents the mean from analyses of 4 tissue pools of at least 2 birds per pool. Statistical comparisons are described in Table 2.

† Values in parentheses are corresponding relative percentages of the total lipid.

Table 2—Statistical Analysis of Lipid Data Presented in Table 1 for White Carneau (WC) and Show Racer (SR) Celiac Foci

	Age comparisons			Breed comparisons				Aortic site comparisons (Tables 1 and 3)			
	1 day–6 weeks	6 weeks–12 weeks	12 weeks–6 months	1 day	6 weeks	12 weeks	6 months	1 day	6 weeks	12 weeks	6 months
Cholesterol											
WC	S	NS	S	NS	NS	S	S	NS	NS	S	S
SR	S	NS	NS					NS	S	NS	S
Nonesterified fatty acids											
WC	S	NS	NS	S	S	S	S	S	S	S	S
SR	S	S	S					NS	NS	NS	S
Triacylglycerols											
WC	S	NS	S	NS	NS	NS	S	S	NS	NS	S
SR	S	NS	NS					S	S	NS	NS
Cholesteryl esters											
WC	NS	S	S	NS	NS	NS	S	NS	NS	S	S
SR	NS	S	NS					NS	NS	S	NS
Phospholipids											
WC	S	NS	S	S	NS	NS	S	NS	NS	S	S
SR	S	NS	NS					NS	NS	S	S
Hydrocarbon											
WC	S	S	S	NS	NS	S	NS	NS	NS	S	S
SR	S	S	S					NS	NS	S	S
Total lipids											
WC	S	S	S	NS	S	NS	S	NS	S	S	S
SR	S	S	S					NS	NS	S	S

S = significantly different (*P* < 0.05); NS = not significantly different.

Table 3—Lipid Content of Upper Thoracic Aortas as a Function of Age in White Carneau (WC) and Show Racer (SR) Pigeons

	Cholesterol	Nonesterified fatty acids	Triacylglycerols	Cholesteryl esters	Phospholipids	Hydrocarbon	Total lipid
1 day							
WC	0.18 ± 0.02* (14.8)†	0.07 ± 0.01 (5.7)	0.26 ± 0.02 (21.3)	0.26 ± 0.04 (21.3)	0.24 ± 0.04 (19.7)	0.21 ± 0.05 (17.2)	1.2 ± 0.1
SR	0.19 ± 0.07 (15.4)	0.06 ± 0.02 (4.9)	0.29 ± 0.06 (23.6)	0.18 ± 0.05 (14.6)	0.22 ± 0.04 (17.9)	0.29 ± 0.09 (23.6)	1.2 ± 0.1
6 weeks							
WC	1.21 ± 0.20 (16.4)	1.39 ± 0.21 (18.9)	1.07 ± 0.18 (14.5)	0.19 ± 0.01 (2.6)	1.77 ± 0.34 (24.1)	1.73 ± 0.36 (23.5)	7.4 ± 0.2
SR	0.65 ± 0.13 (11.4)	0.36 ± 0.09 (6.3)	1.45 ± 0.10 (25.4)	0.52 ± 0.09 (9.2)	1.23 ± 0.19 (21.6)	1.49 ± 0.15 (26.1)	5.7 ± 0.4
12 weeks							
WC	0.66 ± 0.20 (10.2)	0.29 ± 0.02 (4.5)	1.74 ± 0.43 (26.8)	0.49 ± 0.13 (7.6)	1.59 ± 0.38 (24.6)	1.70 ± 0.14 (26.3)	6.5 ± 0.3
SR	0.44 ± 0.08 (8.4)	0.88 ± 0.29 (16.7)	1.58 ± 0.48 (30.0)	0.23 ± 0.08 (4.4)	0.51 ± 0.17 (9.7)	1.62 ± 0.17 (30.8)	5.3 ± 0.7
6 months							
WC	0.50 ± 0.15 (4.4)	1.62 ± 0.15 (14.2)	2.97 ± 0.79 (26.1)	1.24 ± 0.36 (10.9)	1.46 ± 0.28 (12.8)	3.59 ± 0.52 (31.6)	11.4 ± 2.0
SR	0.09 ± 0.01 (1.0)	1.23 ± 0.06 (13.9)	2.44 ± 0.44 (27.6)	1.00 ± 0.05 (11.3)	1.04 ± 0.04 (11.8)	3.04 ± 0.14 (34.4)	8.8 ± 0.4

* Values expressed as µg lipid/µg DNA ± SEM. Each value represents the mean from separate analyses of 4 tissue pools consisting of at least 2 birds per pool. Statistical comparisons are described in Table 4.

† Values in parentheses are corresponding relative percentages of the total lipid.

Table 4—Statistical Analysis of Lipid Data Presented in Table 3 for White Carneau (WC) and Show Racer (SR) Upper Thoracic Aortas

	Age comparisons			Breed comparisons			
	1 day–6 weeks	6 weeks–12 weeks	12 weeks–6 months	1 day	6 weeks	12 weeks	6 months
Cholesterol							
WC	S	NS	NS	NS	NS	NS	NS
SR	NS	NS	NS				
Nonesterified fatty acids							
WC	S	S	S	NS	NS	NS	NS
SR	NS	NS	NS				
Triacylglycerols							
WC	S	NS	NS	NS	NS	NS	NS
SR	S	NS	NS				
Cholesteryl esters							
WC	NS	NS	NS	NS	NS	NS	NS
SR	NS	NS	S				
Phospholipids							
WC	S	NS	NS	NS	NS	NS	NS
SR	S	NS	NS				
Hydrocarbon							
WC	S	NS	S	NS	NS	NS	NS
SR	S	NS	NS				
Total lipids							
WC	S	NS	S	NS	NS	NS	NS
SR	S	NS	S				

S = significantly different ($P < 0.05$); NS = not significantly different.

From 1 day to 12 weeks of age, there was little difference between breeds in the general trend toward increased amounts of triacylglycerols, cholesteryl esters, phospholipids, and hydrocarbon in the celiac foci. After 12 weeks, however, absolute amounts of all lipid classes except hydrocarbon were significantly greater in WC celiac foci than in SR foci (Tables 1 and 2). A breed difference in hydrocarbon in celiac foci was detected only at 12 weeks of age.

Finally, it is noteworthy that triacylglycerols and cholesteryl esters are present in proportions similar to those of cholesterol and nonesterified fatty acids in WC celiac foci by 6 months (10–12%), while phospholipids and hydrocarbon constitute the major part of lipid in both breeds at this age.

Aortic Site Differences: Celiac foci (Table 1) have significantly ($P < 0.05$) more total lipid than thoracic segments (Table 3) by 6 weeks in WCs and by 12 weeks in SRs (Table 2).

Cholesterol was significantly greater in WC celiac foci than thoracic aortas at 12 weeks and 6 months of age. Similar differences were seen in SRs at 6 weeks and 6 months of age. Although nonesterified fatty acids were significantly greater in WC celiac foci than in thoracic aortas at 1

day and subsequent ages, a similar difference was not observed in the SRs until 6 months of age. It is noteworthy that an increase in cholesteryl esters was seen at 12 weeks in SR celiac foci; however, in contrast to WC celiac foci, in which cholesteryl esters increased dramatically by 6 months, levels of cholesteryl esters (as well as triacylglycerols) in SR celiac foci remained essentially the same and approximated those in WC and SR thoracic aortas at 6 months of maturation. Finally, phospholipid and hydrocarbon accumulations were more prevalent in the celiac foci than in thoracic areas in both breeds at 12 weeks and 6 months of age.

Profile of Nonesterified Fatty Acids

Since the earliest major breed difference in lipid composition between the celiac foci was seen in NEFA at 6 weeks of age, the nature of the fatty acids was examined at this age and at 6 months.

Summation of total amounts of individual higher fatty acids $(C_{14:0}-C_{20:4})$ confirmed the TLC data that 6-week-old WC celiac foci contained significantly more NEFA than SR celiac foci. This difference was primarily due to the larger amounts of palmitate, stearate, oleate, and linoleate in WCs, compared with SRs (Table 5).

Major age-related trends from 6 weeks to 6 months in WC celiac foci and upper thoracic aortas were increased amounts and proportions of shorter-chain fatty acids (myristic and myristoleic acids) and saturated fatty acids at the expense of unsaturated free fatty acids (Tables 5 and 6). These trends are especially significant, since WC celiac foci had greater quantities of unsaturated fatty acids than SR celiac foci at 6 weeks of age (Table 1). Contrary to these age-related trends in WCs, absolute amounts and proportions of unsaturated fatty acids increased during this age period in SR celiac foci and upper thoracic aortas, while the total amounts in the SR foci more than doubled. Particularly noteworthy is the striking decrease in the unsaturated/saturated and the polyunsaturated/saturated ratios at both WC aortic sites as a function of age (Table 7). The opposite pattern occurred in SR.

Ultrastructure

The raised intimal thickenings at the celiac branch of both breeds at 6 weeks of age were covered by a single layer of endothelial cells characterized by distinct abutting and overlapping junctions (Figures 1 and 2). In some sections, filiform projections or irregularities in the contour of the luminal surface were evident (Figures 1 and 2), but this observation was inconsistent and followed no trend. These cells were always present covering the raised intimal thickenings in both breeds at all ages examined and

Table 5—Nonesterified Fatty Acids in White Carneau (WC) and Show Racer (SR) Aortas at 6 Weeks of Age

	WC		SR	
	Celiac cushions	Upper thoracic	Celiac cushions	Upper thoracic
14:0	Trace (<1%)*	Trace (<1%)	Trace (<1%)	0.01 ± 0.01 (1.6)
14:1	Trace (<1%)	Trace (<1%)	Trace (<1%)	0.01 ± 0.01 (1.6)
16:0	1.65 ± 0.27†a,g (33.2)	0.18 ± 0.02g (21.7)	0.27 ± 0.05a (25.4)	0.18 ± 0.01 (27.9)
18:0	0.69 ± 0.11b,h (14.4)	0.09 ± 0.02h (10.9)	0.14 ± 0.04b (12.8)	0.10 ± 0.03 (15.6)
18:1	2.49 ± 0.16c,i (41.7)	0.46 ± 0.08f,i (56.7)	0.58 ± 0.07c,l (56.0)	0.22 ± 0.06f,l (33.7)
18:2	0.63 ± 0.26d,j (10.5)	0.07 ± 0.01j (6.1)	0.03 ± 0.02d (2.8)	0.07 ± 0.03 (9.7)
18:3	Trace (<1%)	0.03 ± 0.02 (2.6)	Trace (<1%)	0.06 ± 0.03 (9.2)
20:3	Trace (<1%)	Trace (<1%)	Trace (<1%)	Trace (<1%)
20:4	Trace (<1%)	Trace (<1%)	Trace (<1%)	Trace (<1%)
TOTAL	5.47 ± 1.50e,k	0.85 ± 0.18k	1.05 ± 0.11e	0.66 ± 0.03

* Values in parentheses are relative percentages.
† μg NEFA/μg DNA ± SEM. Each value represents the mean from separate analyses of 4 tissue pools of at least 2 birds per pool.
a–l. Values with the same superscript are significantly different (P < 0.05).
a–f. Differences between breeds.
g–l. Differences between aortic sites.
Saturated fatty acids: 14:0 myristic acid; 16:0 palmitic acid; 18:0 stearic acid.
Unsaturated fatty acids: 14:1 myristoleic acid; 18:1 oleic acid; 18:2 linoleic acid; 18:3 linolenic acid; 20:3 eicosatrienoic acid; 20:4 arachidonic acid.

Table 6—Nonesterified Fatty Acids in White Carneau (WC) and Show Racer (SR) Aortas at 6 Months of Age

	WC		SR	
	Celiac cushions	Upper thoracic	Celiac cushions	Upper thoracic
14:0	0.07 ± 0.03* (1.6)†	0.06 ± 0.02 (5.1)	Trace (<1%)	Trace (<1%)
14:1	0.07 ± 0.03 (1.6)	0.07 ± 0.03 (4.9)	Trace (<1%)	Trace (<1%)
16:0	2.40 ± 0.15[a,i] (52.0)	0.72 ± 0.12[e,i] (56.7)	0.48 ± 0.05[a,m] (22.2)	0.23 ± 0.01[e,m] (21.4)
18:0	1.39 ± 0.29[b,j] (29.0)	0.34 ± 0.06[f,j] (27.0)	0.21 ± 0.05[b] (10.1)	0.09 ± 0.01[f] (8.6)
18:1	0.69 ± 0.15[k] (14.3)	0.08 ± 0.03[g,k] (5.7)	0.94 ± 0.16[n] (42.6)	0.47 ± 0.02[g,n] (43.1)
18:2	Trace (<1%)	0.03 ± 0.01[h] (2.0)	0.55 ± 0.09[c] (24.9)	0.28 ± 0.01[n] (26.0)
18:3	Trace (<1%)	Trace (<1%)	Trace (<1%)	Trace (<1%)
20:3	Trace (<1%)	Trace (<1%)	Trace (<1%)	Trace (<1%)
20:4	Trace (<1%)	Trace (<1%)	Trace (<1%)	Trace (<1%)
TOTAL	4.71 ± 0.49[d,l]	1.28 ± 0.23[l]	2.18 ± 0.23[d,o]	1.09 ± 0.04[o]

* μg NEFA/μg DNA ± SEM. Each value represents the mean from separate analyses of 4 tissue pools of at least 2 birds per pool.

† Values in parentheses are relative percentages.

a–o. Values with the same superscript are significantly different (P < 0.05).

a–h. Differences between breeds.

i–o. Differences between aortic sites.

Table 7—Ratios of Unsaturated and Polyunsaturated to Saturated Nonesterified Fatty Acids

	WC		SR	
	Celiac focus	Thoracic aorta	Celiac focus	Thoracic aorta
6 weeks				
Unsaturated: saturated	52:48 (1.08)	67:33 (2.03)	62:38 (1.63)	55:45 (1.22)
Polyunsaturated: saturated	11:48 (0.23)	9:33 (0.27)	3:38 (0.08)	19:45 (0.42)
6 months				
Unsaturated: saturated	17:83 (0.21)	12:88 (0.14)	68:32 (2.13)	70:30 (2.33)
Polyunsaturated: saturated	0:83 (0)	2:88 (0.02)	25:32 (0.78)	26:30 (0.87)

resembled the endothelial cells present in non-lesion areas of the thoracic aorta. A more detailed morphologic investigation of the surface of endothelial cells, employing both scanning and transmission electron microscopy, as well as morphometric analysis of the different anatomic sites, will be the subject of the forthcoming paper.

The space immediately below the endothelial cells was occupied by an electron-dense amorphous basement membrane that contained thin, discontinuous elastic laminas. Closely apposed to the basement membrane were intimal cells recognized as smooth muscle by their complement of myofibrils and dense plaques and cell-surface–associated basal laminas that were markedly thickened in some sections (Figure 2).

In some intimal "pads," the smooth muscle cells were closely juxtaposed and separated from one another by small fragments of elastic tissue and pools of vesicular structures (Figure 1), while other areas of intimal thickening contained smooth muscle cells separated by wide extracellular spaces that were also filled with fragments of elastic tissue, basal-lamina–like material, and extracellular vesicular structures ranging in size from 20 to 700 nm (Figure 2).

By 6 months of age, the intimal thickenings in both breeds contained numerous smooth muscle cells that were separated by extracellular space containing basal-lamina–like material, large pleomorphic membranous sacs, and small vesicular structures (Figures 3 and 4). Collagen and elastica were present but sparse.

The major difference in ultrastructure between the celiac bifurcation site and the unbranched thoracic aorta in these two breeds was the presence of the extracellular vesicular structures and excessive accumulation of basal-lamina–like material between cells. Although no quantitative measurements were done on the extent of this material, the thickenings at

6 months of age always contained more debrislike material than the same sites at 6 weeks, and there was consistently more of this material in the WC celiac cushions than in those of the SR.

There appeared to be two different types of extracellular vesicular structures. Some of the vesicles were uniformly round, ranged in size from 20 to 100 nm, possessed single membranous profiles, and often occurred in aggregates (Figures 1 and 4). Other vesicles were larger, more heterogeneous in shape, stained more intensely with osmium, and occasionally possessed concentric membranous profiles (Figures 2–5). Many of these extracellular vesicles appeared to originate from budding of the smooth muscle cell plasma membrane or from extensive breakdown of parts of smooth muscle cell cytoplasm that had appeared to form thin branches or herniated regions (Figure 5).[37,38]

Although an occasional cell immediately below the endothelium contained intracellular lipid droplets in both breeds, extracellular vesicular deposits were far more common at 6 weeks and 6 months of age.

Discussion

One of the earliest lipid class differences between celiac sites was non-esterified fatty acid accumulation in the WC. Such accumulation during early atherogenesis is consistent with other studies,[39] and several hypotheses may explain this pattern. By 6 weeks of age, WC celiac mitochondria lack control of NADH transhydrogenation by adenosine triphosphate (ATP) (unpublished observation) which may lead to higher $NADPH/NADP^+$ ratios in the cell.[17,40] It has been suggested that this lack of regulation plays an important role in the control of aortic fatty acid accumulation by increasing biosynthesis.[17,41] Superimposed upon this lesser capacity of WC celiac mitochondria to regulate NADH transhydrogenation at 6 weeks of age is a shift in energy production from the tricarboxylic acid cycle (TCA) to glycolysis.[42] Lower TCA cycle activity in WC aortas beginning at 5 to 8 weeks of age also appears consistent with the hypothesis of increased NEFA production, since lower TCA cycle activity would retard fatty acid oxidation and favor synthesis by increasing the availability of citrate (and acetate).

Pathobiologic effects of NEFA accumulation include stimulation of uncoupled mitochondrial ATPase activity,[16,43] an increase in aortic elastolysis,[44,45] and structural damage to the mitochondrion to cause eventual uncoupled respiratory-chain phosphorylation[46] by 6 months of age.[18] Increased palmitate, stearate, or oleate (as found in this study) allow the inner mitochondrial membrane to become permeable to protons.[43] In addition, pools of NEFA in WC celiac foci would favor subsequent

accumulation of esterified lipids, such as cholesteryl esters, phospholipids, and triacylglycerols as cholesterol and glycerol phosphate accumulate.

Smith et al [47] reported that more than 50% of the total fatty acids in embryonic WC aortas were of the saturated variety (myristic, palmitic, and stearic acids). The present study showed a rapid increase in the percentage of saturated NEFA and a decrease in unsaturated NEFA in WC celiac cushions from 6 weeks to 6 months of age. Saturated NEFA (palmitate and stearate) were also shown to be the predominant fatty acids esterified to cholesterol at 6 weeks and 6 months of age in WC celiac cushions.[48]

The increase in saturated fatty acids and the decrease in unsaturated fatty acids at branched portions of WC aortas by 6 months of development may be attributed to lower levels of oxygen found in these foci, beginning at 12 weeks of age,[20] since oxygen is needed for desaturation.[49] As a result, reduced levels of linoleic acid, a precursor for prostaglandin (PG) production,[50] may provide conditions which favor cholesteryl ester accumulation, since lack of these PGs may permit uncontrollable synthesis and/or insufficient stimulation of cholesteryl ester hydrolysis.[51]

Aortic tissue can synthesize saturated fatty acids,[52] elongate fatty acid chains, and desaturate in certain positions.[39] However, this organ cannot produce linoleate;[53] so linoleate in WC probably arises from plasma lipoproteins (containing cholesteryl linoleate) perfusing through the arterial wall.[53,54] Furthermore, it seems unlikely that the saturated (myristic, palmitic, and stearic) and monoenoic (myristoleic and oleic) acids accumulating in WC celiac cushions originate from only one source, since Subbiah et al [55] found these fatty acids to be present in the plasma; and St. Clair et al [52] demonstrated that pigeon aortas synthesize myristic, palmitic, stearic, and oleic acids *in situ*. Consequently, the origin of aortic NEFA remains to be elucidated.

A consistent early morphologic finding at arterial bifurcations in the WC was the progressive accumulation of extracellular membranous material beginning as early as 6 weeks of age (coinciding with significant increases in NEFA) and becoming prominent by 6 months. Some of the large pleomorphic structures resembled cell debris as described by several investigators,[37,38,56-66] while the smaller, more homogeneous vesicles resembled what has been described as extracellular lipid and/or precipitated lipoprotein.[56,57,67-71] The finding of similar debrislike material and smooth muscle cell processes in arteries from hypertensive animals,[61-64,72] as well as in intimal thickenings at arterial bifurcations in young rabbits [56] and human infants,[57] suggests that the appearance of this material may be hemodynamically induced and that these areas may be subjected to re-

peated mild insults. Since this material also appears at bifurcations in SR arteries, but to a lesser extent than at similar sites in WC, these sites may be subjected to the same type of insults, but the cells in SR respond differently. Previous studies [73-75] have shown serum cholesterol levels, α/β lipoprotein ratios, and blood pressures to be the same in each breed at 9 months. Curwen and Smith [19] have shown that celiac bifurcations in the WC contain significantly more glycosaminoglycans at 6 months of age, compared with corresponding sites in the SR, suggesting that the cells which constitute these intimal thickenings respond differently metabolically. Since glycosaminoglycans form an integral part of extracellular matrix [76] and are capable of interfering with the movement of components through the arterial wall, either by steric exclusion or ionic interaction,[77] the greater amount of glycosaminoglycans in the WC may explain why extracellular debris and lipid accumulate in the celiac bifurcation in the WC.

The origin of the membranous extracellular material is unknown, but morphologic data presented suggest that at least some of this material originates from partial dissolution of smooth muscle cell cytoplasm. Similar observations were made by Joris and Majno,[37] who observed thin branches of smooth muscle cells in the coronary artery of rats, breaking into small pieces of membranous vesicles. In addition, Takebayashi [78] presented evidence suggesting that in hypertensive rats arterial smooth muscle cells could lose part of their cytoplasm by focal necrosis, filling the extracellular space with cellular debris. Wiener and Giacomelli [72] also observed that in angiotensin-induced hypertension, arterial smooth muscle cells formed processes that tended to necrose in the rat.

Similar extracellular vesicular structures have been observed in the WC pigeon and to a lesser extent in the SR in older animals (9 months of age), and these morphologic findings were correlated with increased free and esterified cholesterol concentrations in the WC aorta.[23] Lipid differences between the two breeds were not demonstrated prior to 9 months; however, this may have been due to the fact that whole distal segments of aorta were examined instead of specific areas, such as the celiac region. The present study suggests that this debrislike material accumulates before 9 months in the celiac bifurcation and is correlated with increased lipids and glycosaminoglycans.[19] The finding of significantly more phospholipid in WC celiac cushions at 6 months when compared with SR celiac cushions supports the morphologic observation that some of this debrislike material may be of membranous origin.

Finally, there is substantial evidence [79,80] that the intima of human aortas accumulates large quantities of free cholesterol before esterified cholesterol increases. Such a pattern is consistent with the trend observed

in this study and supports previous observations that lipid accretion in pigeons parallels the pattern seen for humans.[13,21] Also, it has been previously proposed [81] that accumulation of intracellular cholesteryl esters can affect the subsequent release of cholesterol from the cell by inhibiting normal secretory processes. A fivefold difference in both cholesterol and cholesteryl ester content between WC and SR celiac cushions was noted by 6 months of age. Perhaps less esterified cholesterol in SRs may permit more efficient release of free cholesterol from smooth muscle cells.

Large amounts of steryl esters in celiac cushions have been demonstrated in several animal species, including human beings, and their accumulation correlates with the severity of the disease.[24,82,83] Differences in cholesteryl ester synthetase and/or hydrolase activity may account for variations in sterol and steryl ester content observed during the period of accumulation when breed differences were detected. Experiments by Bonner et al [84] demonstrated that species resistant to atherosclerosis had higher cholesteryl ester hydrolytic activity than those susceptible to the disease. A similar conclusion was obtained by comparing cholesteryl ester sythetase and hydrolase activities in aortas from WC pigeons with those from SR aortas.[85] Hydrolytic activity exceeded synthesis in the resistant breed (SR), whereas synthetic activity exceeded hydrolysis in the susceptible breed (WC). Accelerated synthesis may be explained by the substantial availability of substrate (cholesterol, NEFA) to produce cholesteryl esters by 6 months of age. Recently, Hojnacki et al [48] demonstrated that the major cholesteryl esters accumulating in WC celiac cushions by 6 months of age were cholesteryl palmitate and cholesteryl stearate; yet Subbiah and Dicke [86] have shown that cholesteryl ester hydrolase activity in WC and SR aortas is much higher when cholesteryl linoleate and cholesteryl oleate are available. Consequently, the accumulation of cholesteryl esters (CE-palmitate and CE-stearate) may be due to less rapid hydrolysis.

The initial period of lipid accumulation occurs before 6 months of age in sites predisposed to spontaneous lesion formation in WC pigeons, and NEFA is the first lipid to appear in excessive amounts. This lipid accumulation is accompanied by increased extracellular, vesiclelike debris and basal-lamina–like material, correlating with the observed increase in phospholipid and previously reported increase in glycosaminoglycans during this age period at this site.

Although the mechanism(s) responsible for these changes are unknown, altered smooth muscle metabolism in WC celiac foci is believed to play a major role. Further studies of arterial wall metabolism during early spontaneous atherogenesis should elucidate the major mechanism(s) respon-

sible for accumulation of the observed extracellular lipid at these lesion sites during the early stages of the arteriopathy.

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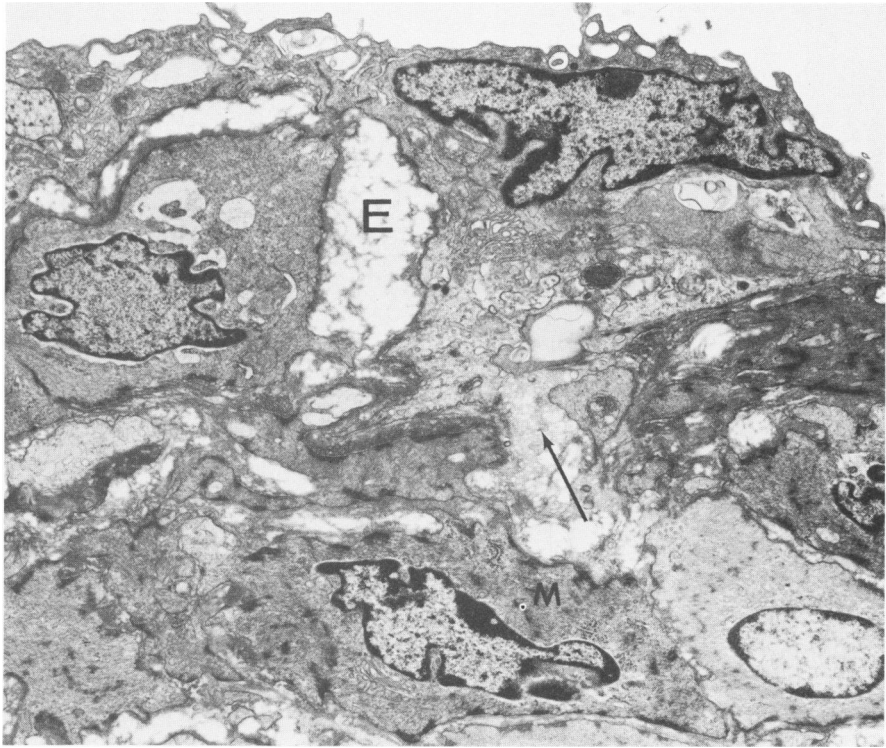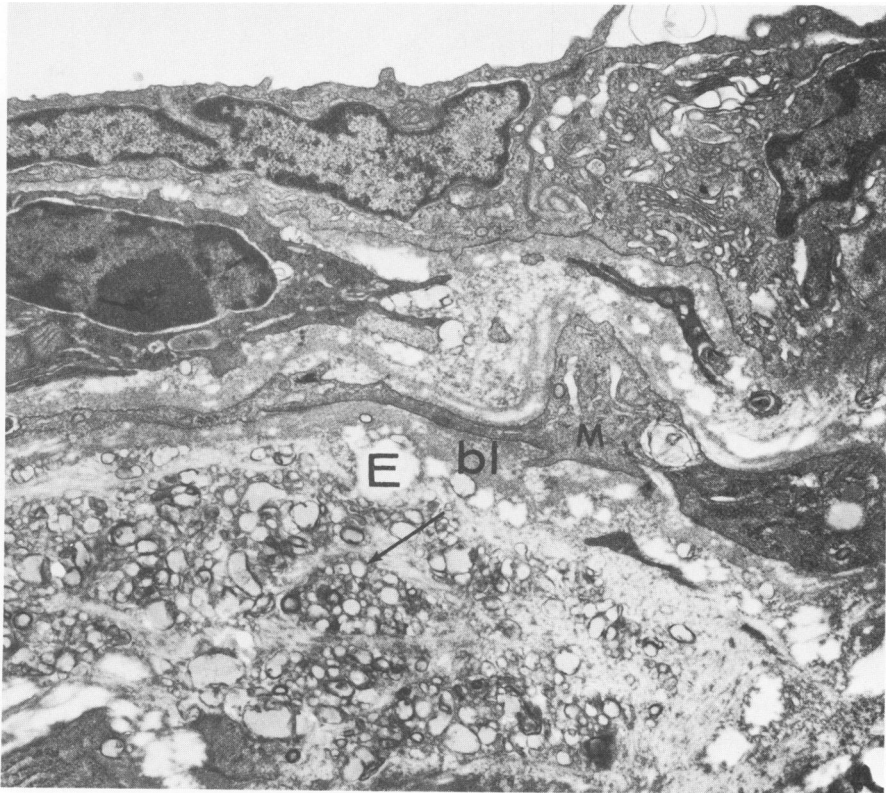
Figure 1—Portion of a celiac cushion from a 6-week-old Show Racer pigeon. Numerous smooth muscle cells are closely packed (*M*), separated by small fragments of elastic tissue (*E*) and occasional "pools" of vesicular structures (*arrow*). (×9000)　　　**Figure 2**—Portion of a celiac cushion from a 6-week-old White Carneau pigeon. The cushion contains a number of modified smooth muscle cells (*M*) with markedly thickened basal laminas (*bl*). The cells are separated by wide intercellular spaces containing abundant debrislike material (*arrows*) and small fibers of elastic tissue (*E*). (×14,250)
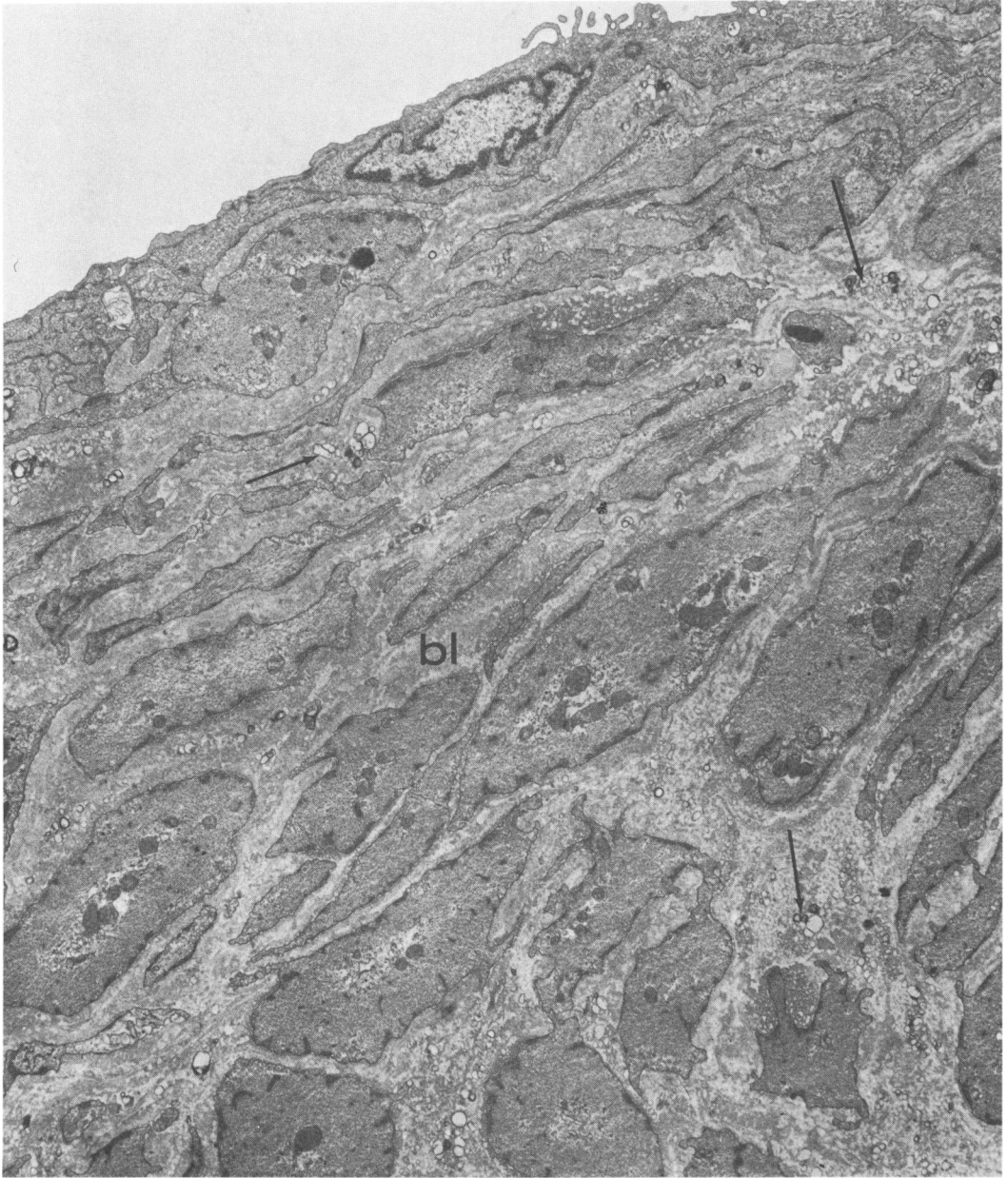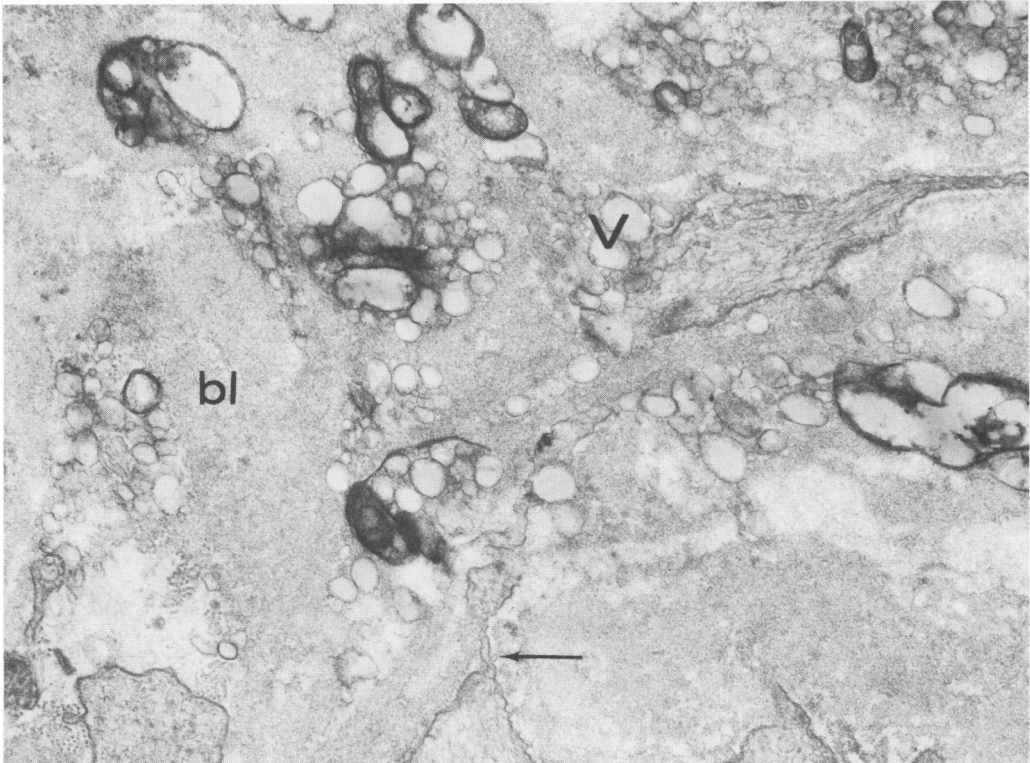
Figure 3—Several layers of smooth muscle cells constitute the 6-month-old celiac cushion in a White Carneau pigeon. The cells are separated by a marked accumulation of basal-lamina–like material (*bl*) and numerous pleomorphic membranous sacs resembling debris (*arrows*). (×9000)
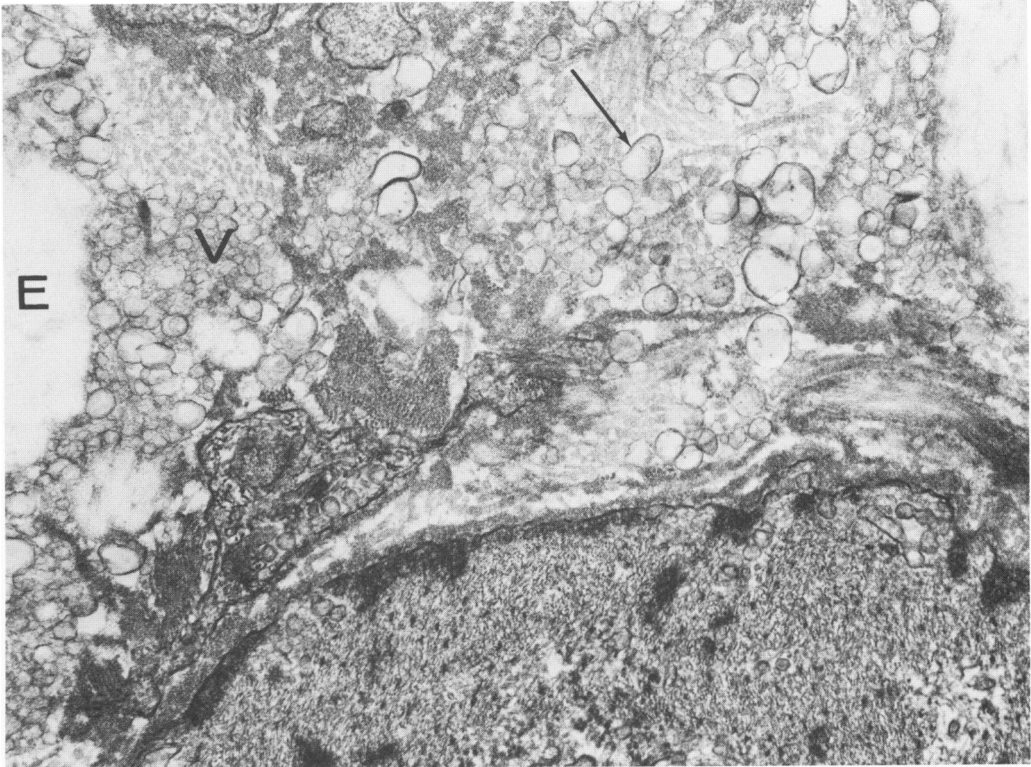
Figure 4—Portion of the intercellular space of 6-month-old White Carneau celiac cushion. This space contains large pleomorphic membranous sacs (*arrows*) and small aggregated vesicular structures (*V*), often associated with elastic fibers (*E*). (×34,000) **Figure 5**—Portion of a thickened intima from 6-month-old White Carneau celiac cushion. Many of the smooth muscle cells formed thin branchlike processes of cytoplasm, which appeared to be undergoing lysis and breaking into membranous vesicles (*V*). Portions of the plasma membranes of these cells formed budlike extensions (*arrow*). The intercellular space also contains abundant basal-lamina–like material (*bl*). (×34,000)

[End of Article]