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Spatial dispersion of stainable lipid in frozen sections of human aorta

R.E. Tracy [1], G.E. Kissling [2], M. Gandia [3], and C. Reynolds [4]

[1] LSU Medical Center, Department of Pathology, 1901 Perdido Street, New Orleans, LA 70112, USA
[2] University of North Carolina, Department of Mathematics, Greensboro, NC 27412, USA
[3] LSU Medical Center, Department of Pathology, 1901 Perdido Street, New Orleans, LA 70112, USA
[4] Tulane Medical Center, Department of Medicine, 1700 Perdido Street, New Orleans, LA 70112, USA

Summary. Frozen sections of human lateral thoracic aorta were stained for lipid with Oil red O. Intensity of staining was judged upon a scale of 0 to 5 brilliance units by matching to a scale of photographs. Microregions, defined by an eyepiece grid, were structured in layers of 100 μm thickness and 1000 μm along the aortic length. The intensity of staining, both total and extracellular, tended significantly to increase with age, intimal thickness and depth into the intima. Aortas having at least one instance of atheronecrosis tended to show generally greater staining intensities than aortas without necrosis, and the tendency was more conspicuous with total than with extracellular staining, implying a greater degree of intracellular staining in the specimens with atheronecrosis. The evidence seems to suggest that the emergence of a necrotic core happens, at least in part, because the affected site has collected an excess of lipid. The evidence further suggests that lipid deposits become excessive with increasing frequency after age 35 years, and that this is associated with intimal thickenings coming to exceed 300 μm. The causes that propel the intimal thickenings remain wholly unknown – those causes seem to have little if anything to do with excess of lipids. Some as yet undetermined qualitative characteristic of the lipids might be of importance in this regard.

Key words: Atherosclerosis – Aging – Pathology

Introduction

The staining of frozen sections with Oil-red-O and other fat stains has been used for many decades in the study of atherosclerosis. Much of the current thinking about the pathogenesis of the disorder derives from the study of such preparations. The areas seen in tissue sections to be affected by various intensities of staining are usually thought to offer information about the lipid composition of the tissue. No quantitative morphometric system has been developed for lipids as it has for DNA, RNA, and other substances. In a filter paper system, the amount of lipid is proportional to the staining intensity when read by spectrophotometer (Swahn 1953). To make this principle quantitative, when judging the intensity of staining by eye is, however, not entirely straightforward. The scheme reported here has evolved slowly over a period of more than 20 years through the collaboration of several investigators (Restrepo and Tracy 1975). This scheme incorporates a strong element of judgement by the observer, and so is not objective in the way that a computerized image analysis would be. Some useful information can nevertheless be obtained from these widely used traditional techniques.

The principal question examined in this study was how fat staining is dispersed through the depths of the aortic intima at various ages and intimal thicknesses. The matter has not been previously pursued in a quantitative way, using a grid of lines in the microscopic field to structure the observations. Without this structuring, visual impressions can be deceptive. The deception arises from two major sources: (a) foam cells tend to collect in the superficial layers (Tracy et al. 1983b), and (b) atheronecrosis has a tendency to avoid the superficial layers of the intima (Tracy et al. 1979). These qualitative differences among the layers could give a false impression about the quantitative dispersal of lipid staining. Hence, the present study used an eyepiece grid to define layers to be evaluated independently.

Offprint requests to: R.E. Tracy

The amount of lipid found in the aortic intima is thought to reflect the blood lipid (Freedman et al. 1988; Smith 1974) and blood pressure (Smith 1974) levels. Those well known observations would favor the view that excessive lipid could be driven into the intima by these factors in the blood. On the other hand, the amount of lipid in the aortic intima seems to be governed by the bulk of interstitial matrix materials (Malcolm 1984; Tracy et al. 1987b). The holding capacity of the intimal bulk may determine its lipid content, with only minor variation in response to factors in the blood. In such a system, the meaning of "excessive" lipid would be hard to define. Clearly, the distribution of microscopic lipid in the intimal spaces would be central to a search for such a definition. One must know what is usual before inquiring about what is excessive. The present report is offered as a beginning toward trying to describe the necessary principles of distribution.

Materials and methods

Selection of cases. The International Atherosclerosis Project (IAP), conducted in 1960–64, brought together in New Orleans about 23,000 sets of coronary arteries and aortas from 14 locations, 5 of them biracial, 19 location-race groups altogether. These vessels were collected from all autopsies performed in those locations during the study period. In a previous study, 686 aortas from 8 of these populations, representing ages 15 to 69 years, were examined microscopically (Tracy et al. 1986). The 8 population groups chosen from the collection represent the extremes of the gradients for observed arterial involvement by raised lesions and fatty streaks. For this study, preparation of frozen fat stains from these 686 specimens was begun in the same sequence of computer-generated random numbers that had been assigned for the H & E stained paraffin sections; 280 aortas were sampled, but only 123 specimens were successfully completed at the conclusion of the study. Attrition of cases because of technical problems was severe. Fifty-six percent of the 280 aortas were unsuitable for complete sampling because of poor dissection, fixation or storage. The greatest loss was from drying during storage, which precludes the preparation of frozen sections. Cases from all eight of the location-race groups were combined into the pool of 123 completed specimens; statistical justification for this pooling of cases is given in the statistical appendix.

Processing of tissues. Aortas were previously opened longitudinally, formalin fixed, sudan stained, and stored in formalin filled plastic bags. The effects upon ORO staining of frozen sections by these previous manipulations, especially the prior Sudan staining, cannot be easily assessed, although most advisors did not consider this to be a major problem. Thoracic segments were newly sampled along the left and right lateral walls from the 4th to the 12th intercostal ostia, as detailed elsewhere (Tracy et al. 1983a). These lateral wall samples were cut into segments, blocked in paraffin, sectioned at 11 μm, and stained with hematoxylin-eosin. Four longitudinal segments of 2 cm length were taken from the left and right lateral walls at the 6th and 10th intercostal levels for frozen sectioning. Frozen 20 μm sections were stained with Oil red O-hematoxylin to reveal lipid in red against a blue background. Stained sections were marked at the middle and two ends to define intervals of 1 cm, 12 marked positions in each aorta. Many of the tissue samples were cut in duplicate sections for staining at separate times. Little variation in the ORO staining intensity was noted between batch-runs, and quantitation of this variability did not seem necessary.

Evaluation of H & E stained slides. The entire left and right lateral wall sample was examined. If any instance of atheronecrosis was found, then the specimen was called "Yes-A"; otherwise it was "No-A". Atheronecrosis is recognized as a necrotic core having masses of plaque gruel intensely stained by ORO, absence of cell nuclei, and cholesterol clefts easily discerned under the 40X objective lens. For purposes of operational definition, greatest interobserver agreement has been attained by relying upon the recognition of cholesterol slits (unstained by ORO) for designating a necrotic core (Tracy et al. 1983a).

Evaluation of fat stains. Under the 10X objective lens, an eyepiece grid marked the intima into layers of 100 μm thickness and 1 mm length; these areas are called "microregions." The deepest layer was treated as whole if over one-half of its area was occupied by intima at the intima-media junction. Uncertainties in the placement of the junction are important sources of method error, as discussed in the next section on reproducibility of measurements. The entire area of each layer as a whole was compared to an array of photographs representing a scale of staining intensity of 0 to 5, and the nearest match was recorded. For want of a name, these units of measure are called "Brilliance Units". A distinction between intracellular and extracellular lipid can often be easily made, whereas at other times the distinction is difficult or impossible. The convention adopted here has been to make a choice at every examined microregion even when the confidence in the choice is limited. The percentage of stain judged to represent extracellular lipid was recorded to the nearest 10%. The product of the total intensity times the extracellular fraction is the extracellular severity grade. These methods are detailed elsewhere (Restrepo and Tracy 1975). Sites with atheronecrosis frequently fell apart during sectioning; to maintain consistency, all sites with necrotic cores were excluded from the grading. This grading scheme calls for a two stage series of subjective decisions; first an impression of total staining intensity is recorded, and then an estimate of percent extracellular is made. The observer error is compounded by taking the product of one observation times another to obtain values for intensities of intra- and extracellular staining. It seems likely, therefore, that the estimates for total staining intensity may be somewhat more precise than those for the separate compartments.

Reproducibility of measurements. To determine how well the observations could be reproduced upon replicate measurement, the right halves of 17 cases were graded a second time by the same observer. A major source of discrepancy between the first and second set of observations upon the same slides was the problem of selecting precisely the same spot both times. Frozen sections are frequently unsuitable for grading because they are torn or folded; the protocol therefore calls for identifying the artefact-free spot nearest to the position marked for measurement. The chosen spot is sometimes not the same from one grading session to the next. Thus, the number of observed microregions was exactly the same in only 65 of the 102 marked positions, suggesting that the intimal thickness was not determined to be exactly the same on two separate tries 36% of the time. An internal elastic lamina is not always well defined,

Table A1. Descriptive statistics for 5 variables measured on 3169 microregions

Variable	Symbol	Mean	SD	Correlation coefficients			
				θ	D	T	E
Age	A	50.2	15.2	0.461	0.320	0.354	0.407
Thickness (Layers)	θ	3.69	2.41	–	0.696	0.430	0.410
Depth	D	2.35	1.73		–	0.374	0.505
Total stain	T	1.91	1.53			–	0.790
Extracellular stain	E	1.44	1.32				–

Age in years; Stain in Brilliance units; thickness is in 100 μm units; Depth is 1 at distances of 0–99 μm from the lumenal surface, 2 at 100–199,..., and 9 at 800–899 μm

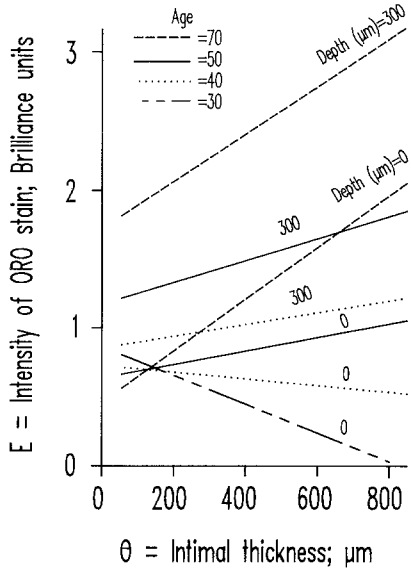


Fig. A1. The equation using the complete model with y = 0 to predict extracellular staining intensity is plotted for depths of 0 and 300 μm at selected values of age. The line for depth = 300 μm at age 30 was constant at E = 0.58 for all thicknesses; it is omitted to avoid over-crowding of the graph

and the placement of the intima-media junction is often a subjective decision based upon pattern recognition, thus accounting for this magnitude of error. Among the 17 cases, the numbers of levels observed at two sessions had a correlation of 0.969. Out of 254 microregions that were observed twice, the intensity of ORO staining differed between replicate readings by 0, 1, 2, and 3 brilliance units in 56, 33, 8, and 2 percent of instances respectively. For the percentage of staining judged to be extracellular, the difference between replicate observations was <10, 10–29, 30–49, and 50–69 percentage units in 57, 25, 11, and 4 percent of instances respectively. Correlation coefficients were 0.81 and 0.71 for replicate measures of intensity and percent extracellular, respectively. For subjective scales of measure based upon the observer's impressions about what is seen, these amounts of method error seem acceptable, especially so since precisely the same spot cannot always be found each time.

Statistics. Complete data were available on each of 3169 microregions; descriptive statistics are given in Table A-1.

Multiple regression models were constructed to relate variables Total Stain (T) and Extracellular Stain (E), to various assortments of the independent variables Age (A), Thickness (θ), Depth (D), Atheronecrosis present or absent (Y), and their interactions, A*θ, A*D, A*Y, θ*D, θ*Y, D*Y, A*θ*D, A*θ*Y, A*D*Y, θ*D*Y. The variable Y is 1 for Yes-A and O for No-A aortas, and the "*" means to take the product. The R^2 statistic for the complete model, using all variables, was 0.281 for the variable T and 0.367 for E. A graph of the equation for the variable E, when related to the complete list of independent variables, is given in Figure A1, with Y = 0. This graph demonstrates the chief characteristics of the equation. When sites of various thickness are compared for any selected age over 40 years, the intima shows greater intensities of ORO staining in the thicker sites, and this tendency is manifest at any specified depth into the intima. The only exception is the immediate subsurface tissue (Depth = 0). Younger than age 40, the reverse is seen: intensity of staining at specified depths decreases with increasing intimal thickening. The importance of these relationships, however, is ambiguous. The variable θ, representing intimal thickness, can be eliminated from this equation with only a tiny decrease in R^2 from 0.367 to 0.355 resulting from its elimination. Because θ and D are strongly correlated (R = 0.696), one variable can often substitute for the other. It is therefore unclear from these data whether thickness, depth, or both are important biologically in the governance of staining intensity.

Attempts were made to simplify these models for T and for E by eliminating one of the independent variables, either Y, A, θ, or D. Removing all terms in Y, the respective R^2's became 0.251 and 0.351. Removing terms in A yielded R^2's of 0.232 and 0.296. Removing terms in θ yielded R^2's of 0.244 and 0.355. Removing the terms in D yielded R^2's of 0.257 and 0.262.

For the variable E, removal of terms in A or D, but not θ, caused serious deterioration in R^2, showing that a simplified model would require A and D but not θ. For the variable T, a simplified model that removes θ was also found to decrease the R^2 only slightly. For consistency, the terms in θ were removed from the models for both variables. Having done this, no other independent variables could be excluded without serious deterioration of the R^2. The resulting models were:

$$E = 0.73 - 0.000163\,A - 0.426\,D - 0.301\,Y + 0.0141\,A*D + 0.0121\,A*Y + 0.448\,D*Y - 0.0092\,A*D*Y \quad (R^2 = 0.355) \quad (1)$$

$$T = 1.19 + 0.000469\,A - 0.428\,D - 0.131\,Y + 0.0127\,A*D + 0.0144\,A*Y + 0.572\,D*Y - 0.0112\,A*D*Y \quad (R^2 = 0.244) \quad (2)$$

These equations are drawn in Figs. 1 and 2 with Y = 0, and in Fig.3 with Y = 1. It should be kept in mind that D is standing in part for θ, and that the elimination of θ is done for convenience. Whether θ is biologically unimportant remains undecided.

Residuals from Equ. (1) and (2) showed no significant differences among the eight location-race groups by one-way ANOVA; this result justifies pooling of cases from all eight sources for analysis of the residuals.

Table 1. Mean and standard error of the mean intensity of ORO staining in 3169 microregions from 123 aortas by age

Age	ORO Intensity[a]				Number of microregions	Average number[b] of levels	Number of aortas
	Total		Extracellular				
	Mean	SEM	Mean	SEM			
10–19	0.89	0.14	0.47	0.07	72	1.0	6
20–29	1.39	0.07	0.75	0.04	357	1.3	26
30–39	1.31	0.06	0.89	0.05	399	2.3	18
40–49	1.47	0.06	0.99	0.05	398	1.9	17
50–59	1.94	0.05	1.57	0.04	787	2.4	25
60–69	2.56	0.05	2.03	0.04	1156	2.9	31

[a] Units of measure (Brillance units = Bu) are from averaging of digits 0 to 5 on an ordinal scale
[b] A level is up to 100 μm thick, counting from the luminal surface to the intima-media boundary; intimal thickness in μm is 100 × number of levels. A microregion is a "level" of 1 mm length along the aorta; at each examined site, the number of microregions equals the number of levels

Results

The objectively assessed total intensity of staining by Oil red O in frozen sections, measured in Brilliance units (Bu) on a scale of 0 to 5, generally tended to increase with age, intimal thickness, and depth into the intima. Taking the difference between the objectively assessed total staining and the roughly approximated estimate of extracellular staining as a reflection of lipid-rich cells, the estimated intracellular staining was inferred to show a generally unchanging trend with age, and tended to decline into the depths of the thick intimal sites. The aortas with at least one instance of atheronecrosis (Yes-A) tended to have conspicuously greater total staining; and the indications were that this trend affected both intracellular and extracellular staining.

Intensity of staining and age. The intensity of staining in nonnecrotic sites increased with age along with increases in the average nonnecrotic intimal thickness (Table 1). For total staining measured in brilliance units (Bu), the rise was from 0.89 Bu to 2.56 Bu from ages 10–19 to 60–69 years. Estimates for extracellular staining indicated a comparable rise from 0.47 Bu to 2.03 Bu. The difference between these measures was nearly constant at about 0.5 Bu for all ages, implying little or no change in the intracellular component.

Intensity of staining and depth into the nonnecrotic intima. The intensity of staining increased with increasing depth into the intima, after exclusion of sites with atheronecrosis (Table 2). For total staining, the increase was from 1.47 Bu at depths of 0–99 μm from the intimal surface to 4.13 Bu at 800–899 μm deep. Estimates of intracellular staining, reflected in the difference between total and extracellular, declined from 0.55 Bu at the first level of depth to 0.23 Bu at the ninth level. The deeper levels tended to be from somewhat older aortas than were the superficial levels (Table 2).

Table 2. Mean and standard error of the mean intensity of ORO staining in 3169 microregions from 123 aortas by depth into the intima

Depth into the intima	ORO Intensity				Number of micro-regions	Average age
	Total		Extracellular			
	Mean	SEM	Mean	SEM		
0– 99	1.47	0.04	0.92	0.03	1309	44.7
100–199	1.84	0.05	1.32	0.04	825	52.3
200–299	2.23	0.07	1.77	0.06	427	51.3
300–399	2.43	0.10	2.08	0.09	235	56.1
400–499	2.73	0.13	2.40	0.13	142	57.9
500–599	3.13	0.17	2.96	0.17	93	60.0
600–699	3.60	0.18	3.45	0.19	65	61.3
700–799	3.60	0.22	3.44	0.23	41	59.6
800–899	4.13	0.21	3.90	0.24	32	60.6

Intimal thickness is in μm; ORO Intensity (Bu) is on a scale of 0 to 5, see footnote Table 1

No-A: Aortas without atheronecrosis. The increase of staining that was seen with increasing distance from the intimal surface was most striking in the aortas from the oldest subjects, younger subjects showing little or no such tendency. This was true for extracellular and for total staining. In Figs. 1 and 2, the data have been presented in a form that was smoothed via regression equations (statistics). The increase of staining that accompanied

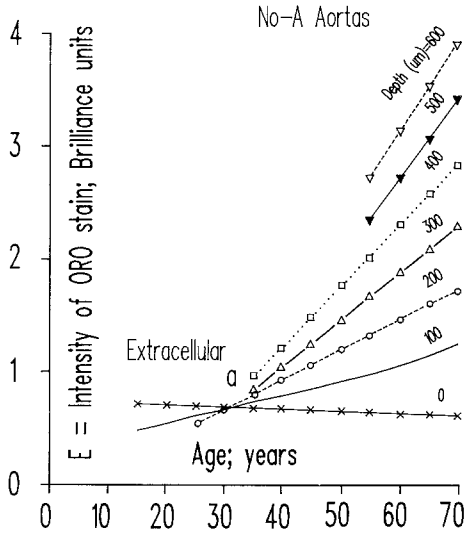


Fig. 1. Equation (1) is plotted with Y = 0. Intensities of extracellular staining predicted from the equation with various values for age and depth into the intima are shown. The position representing intensity 1.0 Bu at age 32 years is marked "a" to aid discussion. Symbols upon the lines are not plotted data points, but only serve to distinguish the lines from each other

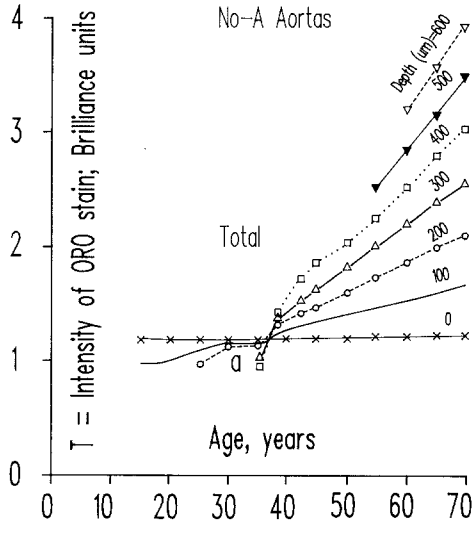


Fig. 2. Equation (2) is plotted with Y = 0. Intensities of total staining predicted from the equation with various substituted values for age and depth into the intima are shown. The position representing intensity 1.0 Bu at age 32 years is marked "a" to aid discussion. Symbols upon the lines are not plotted data points, but only serve to distinguish the lines from each other

aging was more impressive at greater depths into the intima; little change was seen from ages 10–19 to 50–59 years in the most superficial 100 μm of the intima (0.9 Bu to 1.3 Bu for total, and 0.5 Bu to 0.8 Bu for extracellular staining). These patterns were disrupted by a peculiar excess of staining at ages 20–29 years followed by a deficiency at ages 30–39 years, a disruption that causes the curves in Fig. 2 to dip downward around age 35. This disruption was less clear for extracellular than for total staining, which may indicate that only intracellular staining was involved in this aberration that occurred around age 30 years.

The diagram based upon data that were smoothed by a regression equation imply that a nearly constant average extracellular staining intensity of about 0.6–0.7 Bu prevailed at all ages for the tissue immediately in contact with the luminal surface (line labeled "0" in Fig. 1). Deeper levels of intima showed less staining than shallow levels prior to age 30 years, but more staining after that age. Total staining (Fig. 2) showed a pattern much like that for extracellular staining; the main difference is an across-the-board elevation of the lines which depict superficial levels of intima. One consequence of this added increment of staining is to throw a kink into the lines as they pass from age 30 to 40 years. At age 35 years, the depths of 100–400 μm showed little difference between total and extracellular staining, unlike other ages and depths where total exceeded extracellular (compare region "a" in Figs. 1 and 2); this implies a deficiency of intracellular staining in the depths of the newly emerging intimal thickenings as they are first appearing in the 4th decade of life.

Yes-A: Aortas having at least one instance of atheronecrosis. Yes-A aortas also, as in the No-A specimens, showed increasing intensities of staining with age and with depth into the intima for both total and extracellular staining (Fig. 3). Yes-A aortas tended to have conspicuously greater intensities of staining than No-A specimens; the contrast lessened with age and vanished in the depths of elderly aortas, perhaps because of the methodological requirement of discarding the sites with necrotic cores which are too fragile to prepare as frozen sections. Only four Yes-A aortas were under age 40 years, which limits the value of the findings below that age. The crossing over and kinking of lines seen in No-A cases of ages 30–40 years cannot be meaningfully examined in so few Yes-A cases.

Distribution of staining among microregions. The intensity of staining was extremely variable over short distances, sometimes less than 1 mm, in most aortas. Values of zero or maximal could be seen side by side or above and below each other. The frequency distributions in Table 3 illustrate some of the findings in this regard. At depths of 201–500 μm in aortas without atheronecrosis, total

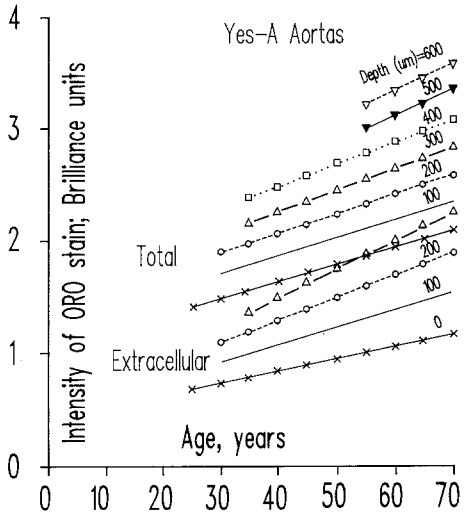


Fig. 3. Equations (1) and (2) are plotted with Y = 1. Symbols upon the lines are not plotted data points, but only serve to distinguish the lines from each other

Table 3. Numbers of microregions with grades 0–5 Bu of total staining intensity by depth into the intima and presence or absence of atheronecrosis

	Intensity of Staining; Brilliance units						Mean intensity
	0	1	2	3	4	5	
Depth = 0–200 μm							
Y = 0	500	482	274	248	83	32	1.4
Y = 1	69	99	114	138	49	46	2.3
Depth = 201–500 μm							
Y = 0	59	109	82	81	31	23	2.0
Y = 1	34	59	84	106	71	65	2.7
Depth = 501–900 μm							
Y = 0	8	7	6	5	9	9	2.6
Y = 1	4	14	19	40	33	77	3.7

Y = 1 if at least one instance of atheronecrosis was found in the entire lateral walls sample, Y = 0 otherwise

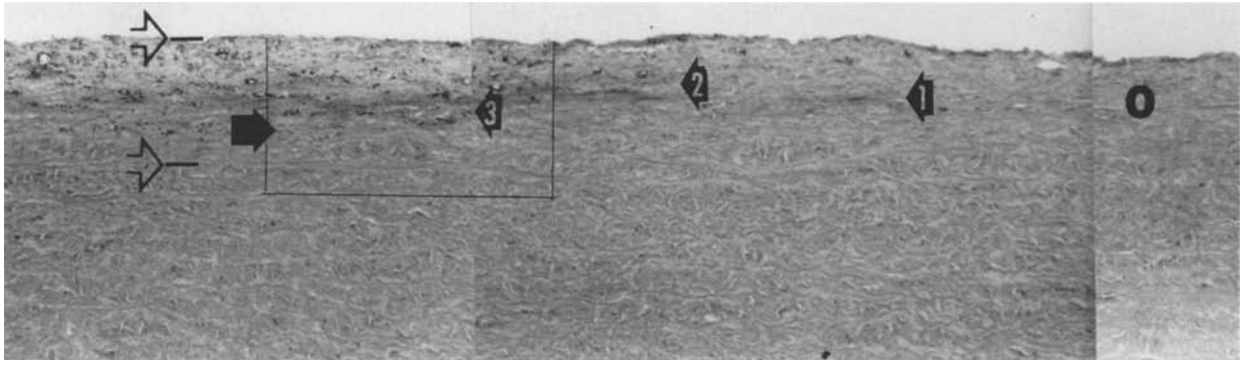


Fig. 4. Representative appearances in the lateral thoracic aorta of a 17-year-old male with mean intimal thickness of 62 μm are shown. Numerals mark areas which illustrate the grading scale of intensities of staining. The heavy arrow marks intima-media boundary. Open arrow heads denote lines that are separated by 100 μm. The outlined area is enlarged in Fig. 5. Frozen-section, ORO, ×200

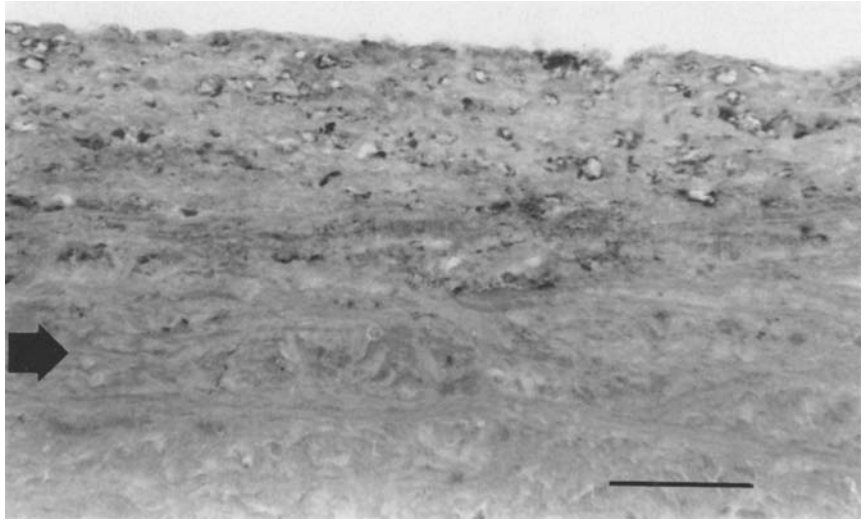
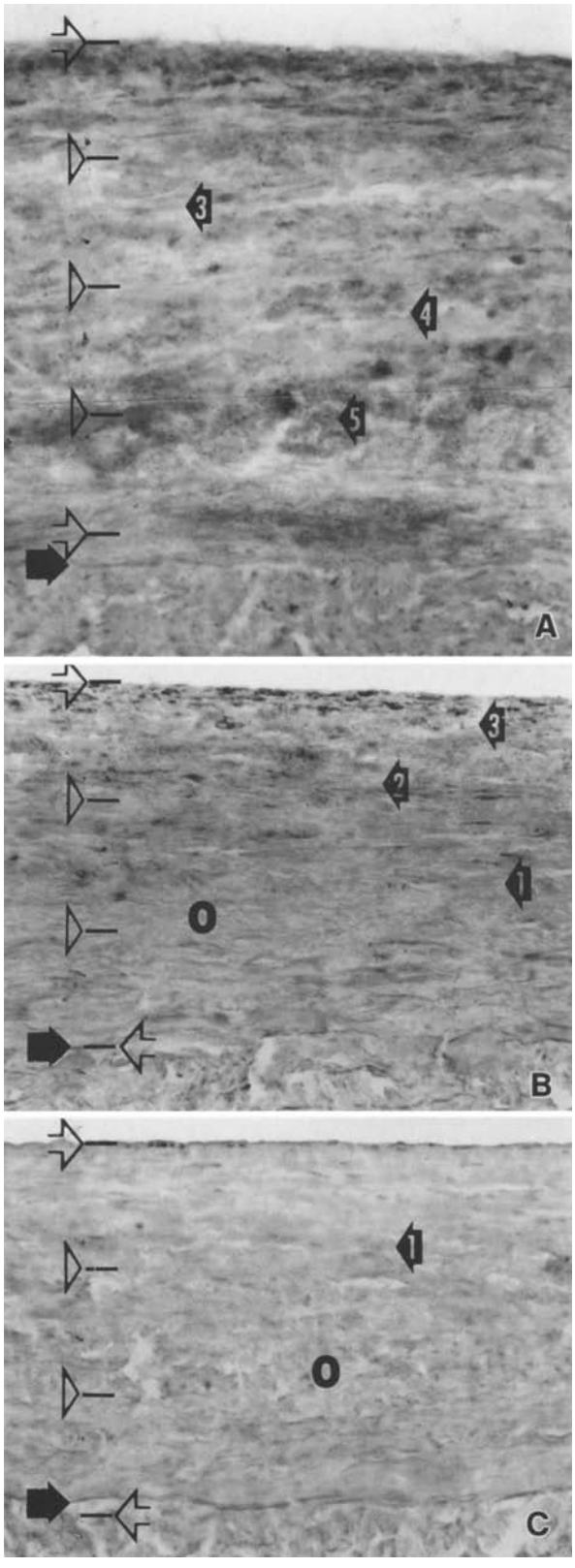


Fig. 5. The outlined area from Fig. 4 is shown at 3× greater magnification. The heavy arrow marks the intima-media boundary. Frozen-section, ORO, ×600



staining intensity averaged 2.0 Bu; but 44% of the microregions were below this value, with gradings of 0 or 1 Bu. Absent or weak staining by technical failure of the stain could conceivably be invoked in some, but certainly not in all 44% of instances, because maximal staining intensity was often present elsewhere in the same sections where instances of zero staining were encountered. Even at depths of 501–900 μm, fully 48% of microregions had gradings no higher than 2 Bu while neighboring regions often had maximal staining; in aortas with sites of atheronecrosis, 20% of the deepest microregions showed intensities no greater than 2 Bu.

Photographic examples. Figures 4 and 5 illustrate some of the usual kinds of appearance that are encountered in aortas of ages 15 to 69 years when the intimal thickness is less than 100 μm. Most of the sample has zero staining, as seen at site "0"in Fig. 4. Elsewhere, grades 1, 2, or 3 are observed in some foci, as shown at sites "1", "2", "3" in Fig. 4; most of this staining is usually intracellular, as in this illustration. Three examples from another aorta illustrate representative appearances in aortas of 300–400 μm intimal thickness at about age 40 (Fig. 6). Staining can be minimal (6C) to maximal (6A) or intermediate (6B) from one place to another in the lateral wall sample, the greater staining intensities tending to be at the thicker sites. Figure (6B), however, exemplifies the "typical" appearance, where the averages given in Figs. 1 and 2 are taken to be "typical". The deeper layers in Fig. (6B) have only extracellular staining, and, although this is only modestly intense, it is brighter than the extracellular staining that is seen in thinner intima (contrast Fig. 4). The greatly thickened intima of elderly aortas often shows minimal to maximal degrees of staining over separations as little as 1 mm, as illustrated in Fig. 7. The necrotic core, although usually lost in preparation, is occasionally prepared adequately; it is always stained to a maximal degree, except where it is calcified (Fig. 8).

Discussion

Intimal thickenings in excess of 300 μm are rare before age 35 years and become increasingly frequent after that age (Tracy et al. 1986). ORO staining intensity was nearly level from ages 15 to 35

Fig. 6. Representative appearances in the lateral thoracic aorta of a 44-year-old male with mean intimal thickness of 320 μm are shown. *Numerals mark* areas which illustrate the grading scale of intensities of staining. *Heavy arrows* mark intima-media boundary. *Open arrow heads* denote lines that are separated by 100 μm. Frozen-section, ORO, ×200

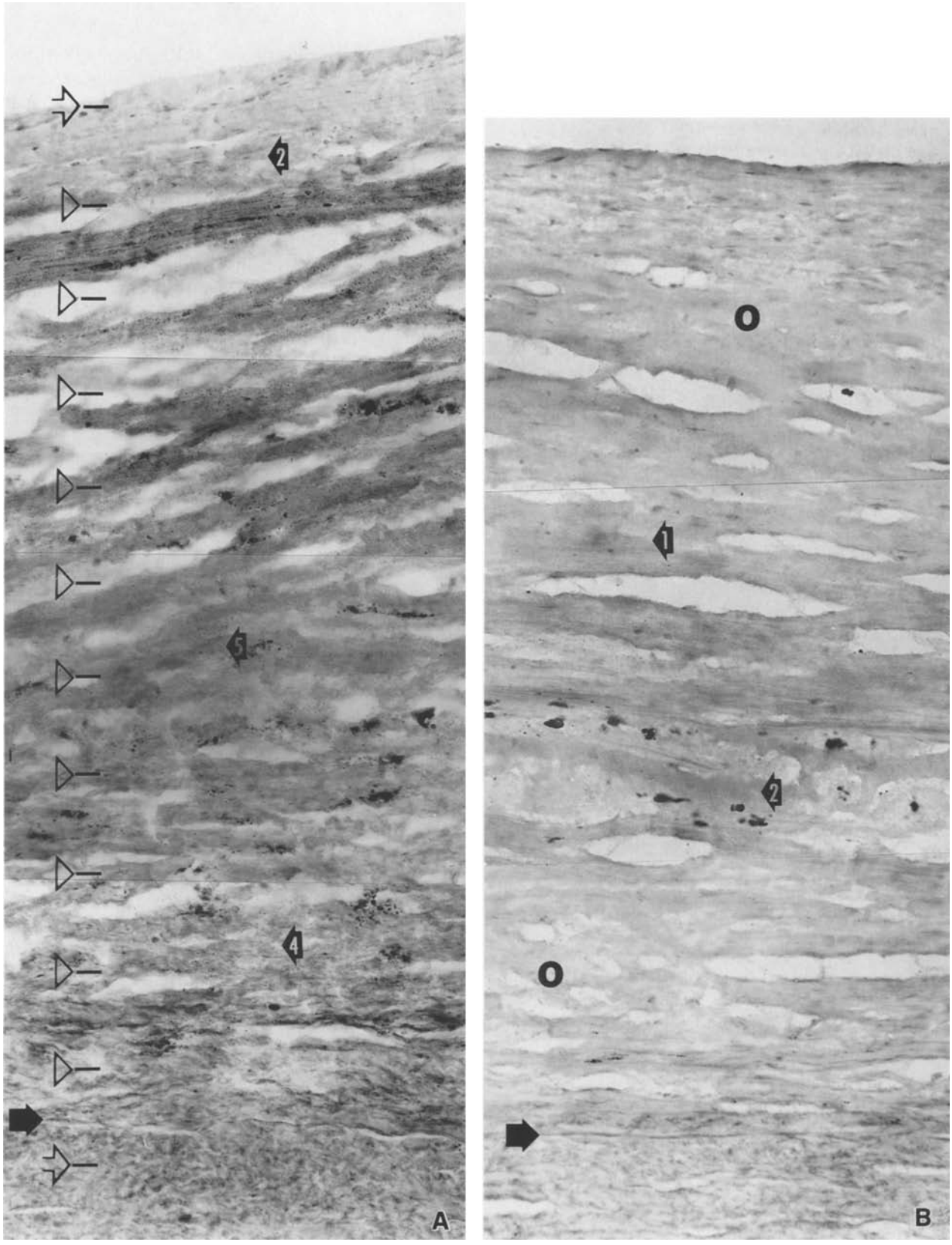


Fig. 7. Representative appearances at some of the thicker sites in nonnecrotic regions of the lateral thoracic aorta of a 68-year-old male with mean nonnecrotic intimal thickness of 406 μm and 31.5% of the specimen affected by atheronecrosis are shown. Numerals mark areas which illustrate the grading scale of intensities of staining. *Solid arrows* mark intima-media boundaries. *Open arrow heads* denote lines that are separated by 100 μm. Frozen-section, ORO, ×200

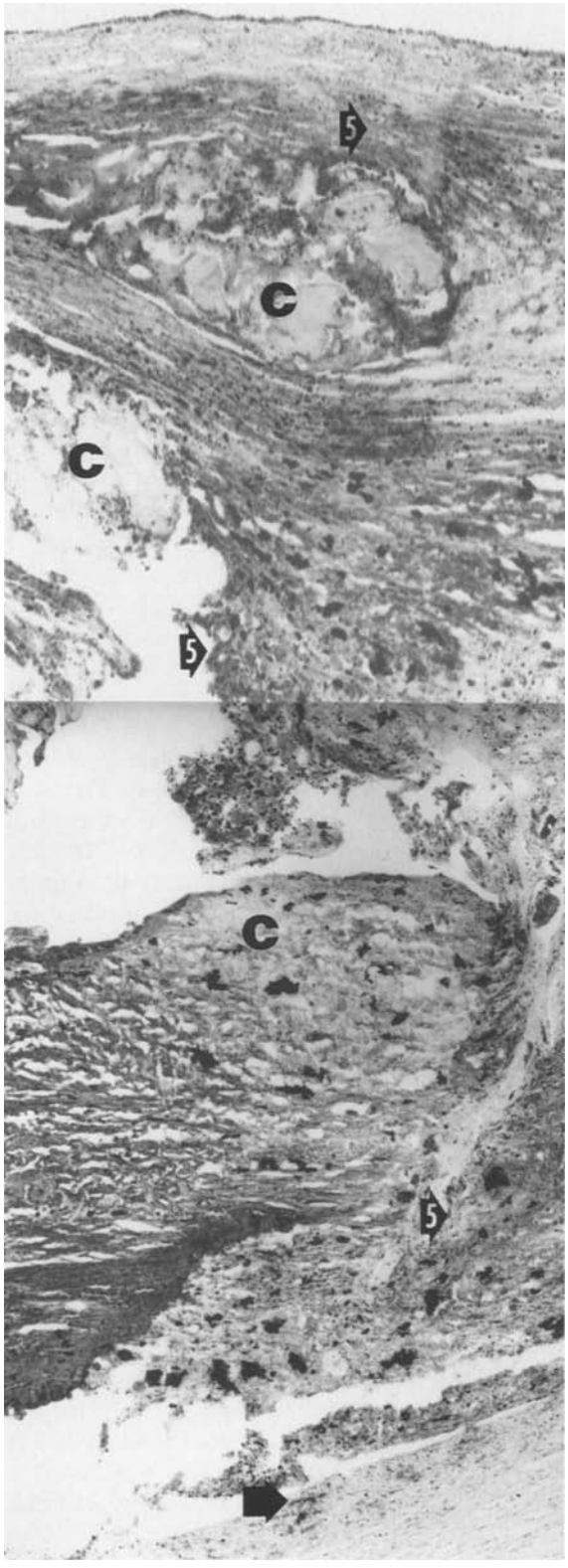


Fig. 8. Calcified ("C") and not calcified examples of atheronecrosis are shown. Staining intensity is maximal (grade 5) everywhere except within the larger mineral crystals. Frozen-section, ORO, ×85

years and increased swiftly with aging after age 35 years. The appearance and progression of intimal thickenings after age 35 years are proposed here to be the initial events that cause the subsequent rising intensities of ORO staining (Figs. 1 and 2). The same conclusion has previously been favored by data obtained from extracting and weighing the lipids from the dissected intima of the abdominal aorta (Malcom et al. 1984); the amount of extractable lipid did not increase swiftly until after age 35 years when the bulk of dry defatted tissue rose to exceed a threshold value (8 mg/cm^2 of surface).

With the first appearance of intimal thickenings around age 35 years, deep intimal tissues at separations of 200–400 μm from the lumen showed weak staining by ORO (cf area "a" in Figs. 1 and 2). The intensity of staining at those intimal depths brightened progressively with age. With further growth of the intima into old age, the intensities of staining increased even in the previously resistant superficial layers of depth 100–200 μm. The results raise the possibility that something in the depths of the aging thicker sites may be favoring the accumulation of lipids, not only in deep repositories, but also in the overlying superficial layers of the vessel wall. Foam cells, ordinarily confined to a superficial position, may sometimes be found in the deep repositories, perhaps because of a chemotactic quality that may arise as the sequestered lipid ages (Bayliss and Adams 1980).

An alternative scenario is that the sites which are in the act of growing thicker may be responding to the lipids with reactive fibroplasia. Three important findings are hard to reconcile with this view. (1) The first lipid deposits at depths of 0–200 μm remain of nearly constant intensity of staining from ages 15 to 30 or 35 years; their intensity of staining only starts to brighten from one age group to the next after the appearance beneath them of new, deeper layers of fibroplasia. (2) Lipid-poor sites as well as lipid-rich ones can thicken after age 35 to 40 years, and lipid-rich sites can fail to do so, as shown in Table 3. (3) Whereas the average intensity of staining rises with age, the rate of intimal growth does not; the growth rate of nonnecrotic intimal thickening is not proportional to the quantity of sequestered lipid from one age group to the next. These results suggest that the quantity of lipids may not be among the factors which propel fibroplasia, but perhaps something in the qualitative composition of the lipids could promote the elaboration of matrix proteins and proteoglycans. These conclusions are in keeping with the reports of Velican and Velican (1983),

who have noted that "fibromuscular" intimal thickenings often precede the appearance of lipid in human coronary arteries.

Not only intimal thickness, but also aging, is having an effect on intensity of staining. This result can be seen in Figs. 1 and 2, although it is shown perhaps somewhat better by the more elaborate multiple regression methods given in the statistics. When comparing sites of equal thickness at equivalent depths, the intensity of ORO staining increases with age, the only exception being the immediate subsurface tissues wherein the staining is the same at all ages. Hence, aging itself seems in some way to enhance the capacity of intimal matrix materials to sequester lipids, perhaps chiefly the extracellular variety, in the remote intimal depths.

Intracellular lipid might seem to be reflected in the quantity that is left by deducting the extracellular from the total staining intensity. It is not necessary, however, to do frozen fat stains to study intracellular lipids; foam cells can be better quantitated in paraffin sections (Tracy et al. 1983b). The results obtained here support the previously reported findings from paraffin sections. Estimates of intracellular (foam cell) lipid show similar patterns in both studies of rising with age and intimal thickness, patterns which include the aberration formed by a peak followed by a slump from ages 25 to 35 years. What is newly seen here for the first time, is that the pattern for the estimates of intracellular lipid, and formerly reported for foam cells, tends to parallel that for the total fat staining in their relationships to age and intimal thickness, but tends to run contrariwise into the depths beyond 300 μm. It seems almost as if the superficial accumulations of foam cells and of deep deposits of extracellular lipid are both responses to the same aging processes which happen in the depths of the thickened intima. Foam cells, however, are seldom seen beyond 300 μm from the lumen until the emergence of a necrotic core (Tracy et al. 1983b). When a necrotic core is present, foam cells can be found at unlimited depths (Bayliss and Adams 1980).

The sites with atheronecrosis are typically composed of necrotic core, fibrous base and fibrous cap (Tracy et al. 1979). Although the core and cap usually cannot be prepared for frozen sections because of their fragility, the occasional successful preparation always showed maximal ORO staining in the necrotic core and in the fibrous base. Variable staining, mostly intracellular, was seen in the cap. The usual fibrous cap was formerly found to have a dense population of foam cells at the cap-atheronecrosis boundary, no matter how thick the cap nor how deeply situated the core (Tracy et al. 1983b). In this study, the aortas which had at least one instance of atheronecrosis were the aortas which generally manifested the greater intensities of ORO staining at all ages, depths, and thicknesses of intima (Figs. 2 and 3). Indications were that this greater intensity of staining was more conspicuous for total than for extracellular staining (Figs. 1, 2, 3). These data generate an impression that foam cells are being drawn to the sites of deep sequestered lipid, and that necrosis tends to emerge where this massing of foam cells is most florid (Stary 1984; Chait 1983). Some aortas had more lipid, but also had evidence for more foam cell response to that lipid – these aortas tended to be the ones with atheronecrosis. A similar phenomenon has previously been reported for the coronary arteries, and the brisk foam cell response to intimal thickenings was found to characterize the subjects with coronary heart disease (Tracy and Kissling 1987). The ability in some persons for deep sequestered lipid to manifest unusually great attraction for foam cells has been brought under suspicion as basic to the events that carry the prone persons toward coronary thrombosis (Tracy et al. 1985).

Nearly every aorta revealed extreme variation in ORO staining from zero to maximal from one place to another in the observed sample. The conspicuous localization of lipids to some sites and not to other sites suggests motion within the intimal compartment of the aorta. Some kind of agitation and stirring could be one possible mechanism for bringing about the observed segregation of lipids. For instance peristaltic actions by pressure waves have been described (Elad et al. 1986). Actions such as these could propel soluble lipoproteins and lipid-rich detritis through the interstitial matrix in a manner that resembles a gel sieve or chromatography column. The thicker sites are clearly favored as gathering places for the fatty detritis, especially in the depths of those sites (Guyton et al. 1985). Some photographs recently published by Orekhov et al. (1987) offer a suggestion for a possible mechanism of the gathering action. Those photographs show early intimal thickenings as humps of dense collagenous tissue sitting upon a deep intimal layer which does not participate in the formation of the hump; lipids gather around and beneath the hump, but tend to be excluded from it. If humps such as this act as obstructions to the axial flow through the intimal compartment, then the lipids that drop from the stream at these sites might, with time, gain the ability to attract foam cells. When the hump itself acquires a population of foam cells, these cells could alter the morphology to obliterate the evidence of pathogenetic origins.

In a recent review of the response-to-injury hy-

pothesis of atherogenesis, Ross (1986) emphasized evidence obtained from experimental animals that had been subjected to various kinds of toxic or surgical insults. This emphasis was justified on the grounds that, "It may never be possible to determine with certainty the specific events that lead to atherosclerosis in humans." The outlook may not be so grim, however, if the challenges of conducting studies in the human are approached with the mathematics of statistical sampling theory. Certain concepts could be layed open for investigation by this approach. The "...importance of intimal smooth muscle cell proliferation as a key event..." (Ross (1986) for instance, has been the subject of only the most cursory attention in human arteries; what little quantitative evidence that is now available tends toward the conclusion that cell proliferation rarely enters the picture until the necrotic core begins to degenerate (Tracy et al. 1986, 1987b). Intimal thickenings in the early stages are due to expansion of interstitial matrix materials (Ross 1986), and this seems to happen with little or no change in smooth muscle cell numbers (Tracy et al. 1987a). The findings with ORO stains reported here offer a way to look at the suggestion that blood lipids deposited into the intima may initiate fibroplasia. The provisional conclusion is that lipids are not usually responsible for the early atherogenic events in the human, and that animal models may be seriously misleading in that regard.

The emergence of necrotic cores in an aorta has been morphologically and statistically associated with maximal intensities of staining by Oil red O. It seems reasonable to guess that necrotic cores happen, at least in part, because the affected sites have collected an excess of lipid. With this meaning of "excess", the data suggest that lipid is rarely excessive before age 35 years. Although the lipid deposits become excessive with increasing frequency after age 35 years, the findings of this study suggest that this increase is a function of intimal thickenings coming to exceed 300 μm. It is these thickenings more than anything else that seem to underlie the excessive deposition of lipids and subsequent atheronecrosis. The causes that propel intimal thickenings remain wholly unknown. The evidence reviewed here suggests that the causes for the growth of fibroplastic intimal thickenings have little if anything to do with excess of lipids in the ordinary course of plaque growth in typical human subjects.

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