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Human Atherosclerosis

III. Immunocytochemical Analysis of the Cell Composition of Lesions of Young Adults

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There have been only limited immunocytochemical studies of the cell composition of the early lesions of human atherosclerosis, and none that incorporate a comprehensive panel of antibodies to various cell types and subsets. The authors thus performed a prospective study of 27 lesions from 16 different individuals ranging in age from 15 to 34 years. These were all lesions that appeared grossly as slightly raised, yellow fatty streaks in the posterior ascending aorta, but on histologic examination had varying degrees of round-cell, spindle-cell, and foam-cell accumulation. Using a panel of antibodies, including monoclonal antibodies specific for smooth muscle cells [HHF35], human macrophages [HAM56], endothelial cells [monoclonal antibodies to F. VIII related antigen], lymphocytes [anti-CD45, anti-CD20, anti-CD45RO, anti–T-cell receptor], it was revealed that the predominant cell type in these early lesions was the smooth muscle cell, including the vast majority of the foam cells, which tended to appear in the deeper regions of the lesions. There were variable numbers of smooth muscle cells and lymphocytes; the latter were exclusively T cells. It is concluded that in atherosclerotic lesions of young adults, which may represent various stages of fatty streak formation and advanced fatty streaks, smooth muscle cell accumulation may be an early event. (Am J Pathol 1992, 140:907–914)

The recent availability of cell-specific monoclonal antibodies that can be used on deparaffinized tissue sections has permitted precise analysis of the cell composition of the lesions of human, nonhuman primate, and rabbit atherosclerosis. By using antibodies that identify epitopes that survive fixation and paraffin embedding, these studies permit preservation of morphologic detail. We recently described the composition of fibrous plaques of human aorta and coronary arteries, confirming that these lesions are composed predominantly of smooth muscle cells and macrophages, together with T lymphocytes and occasional other cell types.[1] Using cell-specific reagents, we were able to demonstrate that both smooth muscle cells and macrophages contributed to the foam-cell population in these lesions. Surprisingly, there have been few immunocytochemical studies that investigated the cellular composition of the early phases of atherogenesis, which precede the development of the fibrous plaque, and in many cases, represent precursor lesions. Using a panel of monoclonal antibodies, a complete cell type analysis of these lesions has been performed.

Materials and Methods

Procurement of Tissue

Grossly appearing fatty streaks were obtained from human autopsy material from the King County Medical Examiner's Office from a total of 27 lesions from 16 different individuals ranging in age from 15 to 34 years. Postmortem intervals ranged from 2 to 22 hours; fatty streaks were identified grossly as characteristic flat- or slightly raised, yellow streaks in the anteriorly incised thoracic aorta. They were immediately excised in the form of post-age stamp-sized segments of aorta and immersion-fixed in methanol-Carnoy's fixative.

Immunocytochemistry

Specimens were fixed overnight, transferred to 100% methanol, and embedded in paraffin. Sections of each

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paraffin block were stained with hematoxylin and eosin, as well as with Verhoeff Van Gieson (VVG) stain for collagen and elastin. In addition, serial sections were incubated with one of a panel of monoclonal antibodies outlined in Table 1. For immunolabeling procedures, either a modification of the avidin-biotin immunoperoxidase method,[1] or a streptavidin-biotin immunoperoxidase method[6] was used. Nickel chloride-enhanced 3,3'diaminobenzidine was used as chromogen and yielded a black reaction product. All immunoperoxidase preparations were counterstained with methyl green. To obtain quantitative data regarding cell type, the total number of antibody-positive cells were counted in each of 27 lesions in the serial HAM56- and HHF35-immunostained sections.

Results

Histology

Fatty streaks, which were identified by their uniform gross appearance, were found, in H&E-stained sections, to be somewhat heterogeneous in cell composition. Most, however, were characterized by intimal aggregates of foam cells containing numerous lipid droplets. In most cases, the lesions were covered by a thin endothelial layer that covered accumulations of round mononuclear cells intermixed with spindle-shaped cells. In addition, many elongated and spindle-shaped foam cells were scattered throughout these lesions, especially in the deeper portions of the lesions. Round mononuclear cells were present in varying numbers in all lesions (Figure 1). Small amounts of connective tissue matrix were deposited around the spindle-shaped cells; however, no dense fibrous tissue, necrotic material, or extracellular lipid was visible by light microscopy in any of the lesions. The intima adjacent to the fatty streaks was diffusely thickened and contained principally elongated spindle-shaped cells.

Immunocytochemical Analysis of Cell Type

The dominant cells comprising the majority of the lesions were HHF35-positive smooth muscle cells, together with large numbers of HAM56-positive macrophages. Quantitative studies (Table 2) revealed that the mean number of smooth muscle cells per lesion was far greater than the corresponding mean number of macrophages (229.7 v. 89.0 cells per lesion); in addition, on average, there were almost twice the number of HHF35-positive foam cells as HAM56-positive foam cells, in the lesions (Table 2). CD45-positive lymphocytes were present; however, these cells represented a variable, but generally small fraction of the total cell population, as depicted in Figures 1–3. HHF35-positive smooth muscle cells were present at all levels (superficial and deep) in the fatty streaks, although the distribution varied from region to region, as described later. The spindle-shaped cells that had accumulated in the subendothelial region of the lesions were all HHF35-positive; the foam cell population located be-

Table 1. *Monoclonal Antibodies Used in Immunocytochemical Analyses*

Clone designation	Specificity	Cells identified in vessel wall	Reference or source	Working dilution
HHF35	Muscle actins	Smooth muscle	1	1:8000†‖
HAM56	*	Macrophages; some endothelium	2	1:2000†‖
2B11, PD7/26	CD45	Lymphocytes, monocytes, macrophages	Dako#	1:80‡‖
L26	CD20	B lymphocytes	Dako#	1:250‡‖
UCHL1	CD45RO	T lymphocytes macrophages	Dako#	1:100‡‖
βF1	T-cell receptor	T lymphocytes	T Cell Sciences**	1:10‡¶
OPD4	CD45RO	T lymphocytes, macrophages	3, 4	1:500‡¶
MAK6	Cytokeratins	—	Triton††	1:50‡‖
35βH11	Cytokeratin 8	—	5	1:1000†‖
F8/86	von Willebrand factor	Endothelium	Dako#	1:50‡‖
Clone 33	Desmin	Smooth muscle	Dako#	1:250‡‖
—	Ulex lectin	Endothelium	Vector‖‖	1:500‡‖

* Antigen incompletely characterized.
† Dilution of ascites fluid.
‡ Dilution of supplied reagent.
‖ Via avidin biotin immunoperoxidase method.
¶ Via streptavidin biotin immunoperoxidase method.
# Dako Corporation, Carpinteria, CA.
** T Cell Sciences, Cambridge, MA.
†† Triton Biosciences, Alameda, CA.
‡‡ Polyclonal antibody (following lectin application).
‖‖ Vector Laboratories, Burlingame, CA.

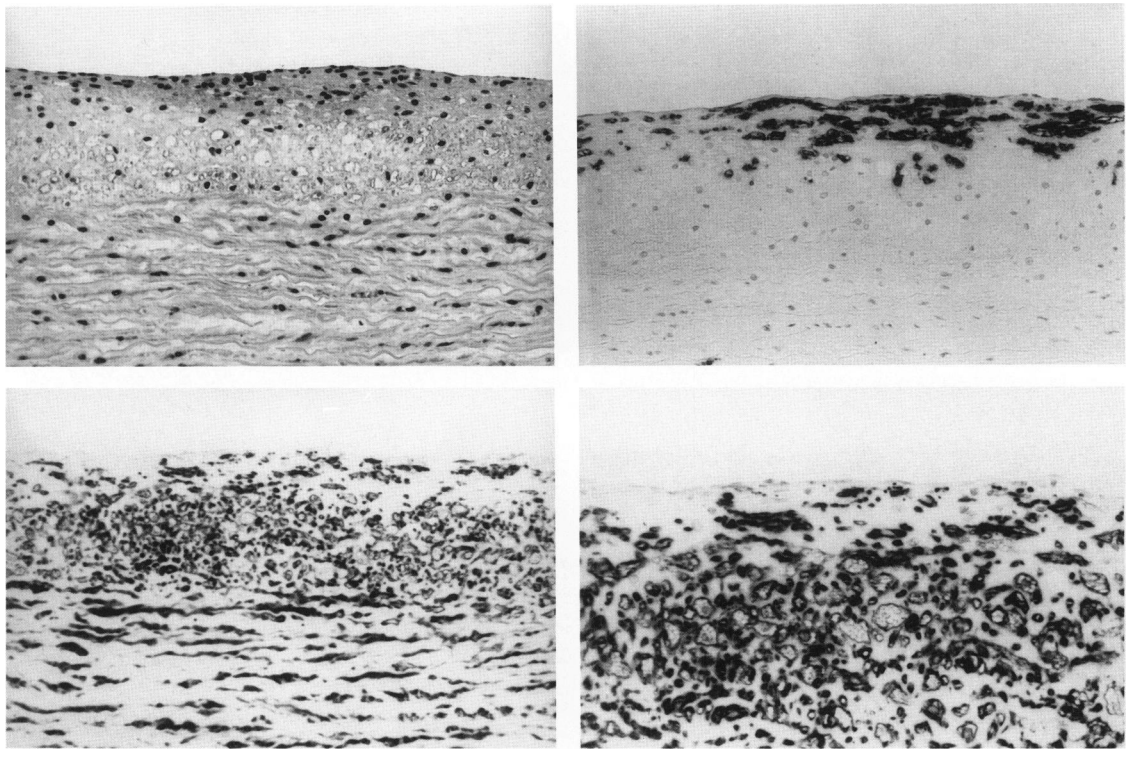

Figure 1. *Sequential sections of a representative early atherosclerotic lesion.* **A:** *Hematoxylin and eosin stain, demonstrating moderate thickness, accumulation of significant numbers of foam cells, especially towards base of lesion, with moderate overall cellularity.* **B:** *Anti-macrophage antibody HAM56 immunocytochemical preparation demonstrating significant numbers of macrophages, confined largely to the more superficial aspects of the lesion; deeper foam cell region is negative.* **C:** *Anti-muscle actins antibody HHF35 demonstrating unexpected cellularity of the lesion, with preponderance of smooth muscle cells.* **D:** *Higher power of HHF35 immunocytochemical preparation demonstrating muscle cell phenotype of foam cell population near base of lesion (***A–C,** *original magnification ×200;* **D,** *original magnification ×400).*

neath these latter cells were also HHF35-positive and HAM56-negative (Figure 1B-D). In general, the HHF35-positive foam cell population appeared directly contiguous with the underlying medial smooth muscle cells (Figure 1C).

HAM56-positive and anti-CD45-positive cells, i.e., macrophages and lymphocytes, tended to be located in the upper half of the lesions (Figures 1B, 2C, 3C,E). A population of subendothelial HAM56-positive mononuclear cells were intermixed with the HHF35-positive cells. HAM56-positive foam cells were rarely seen directly beneath the endothelium, being seen in only 4 of the 27 lesions examined. When present, the HAM56-positive foams cells were larger and more irregularly shaped than HHF35-positive foams cells. When they were found in a subendothelial location, the vast majority of foam cells were HAM56-positive, rather than HHF35-positive, in contrast to the foam cells located deeper in the lesions (Figure 2C,D). In serial sections of lesions, small, but significant numbers, of CD45-positive, HAM56-negative lymphocytes were detected as well, but in fewer numbers than HAM56-positive macrophages (Figures 2, 3). Using several monoclonal antibodies reactive with lymphocyte subsets, it was determined that no B lymphocytes were detectable using the anti-CD20 antibody L26. The lymphocyte population is composed largely, if not exclusively, of T cells, which is supported, as evidenced by reactivity with antibodies UCHL1 and βF1. However, the anti-CD45RO antibody UCHL1 also reacts with a subset of monocytes and macrophages, and the anti-T cell receptor antibody βF1 may not react with all T lymphocytes in the section. Finally, it was determined that the vast majority of these lymphocytes were also OPD4-positive (Figure 3E).

Ulex lectin and anti-von Willebrand factor antibodies

Table 2. *Quantitative Cell Type Data from Early Lesions*

Cell type	Number of cells per lesion*
Macrophages (HAM56-positive cells)	89.0 ± 47.7†
Smooth muscle cells (HHF35-positive cells)	229.7 ± 117.4†
Macrophage-derived foam cells (HAM56-positive)	29.4 ± 22.2‡
Smooth muscle-derived foam cells (HHF35-positive)	55.4 ± 49.6‡

* Each value shows mean ± SD of 27 lesions counted.
† Difference is significant, $P < 0.001$.
‡ Difference is significant, $P < 0.025$.

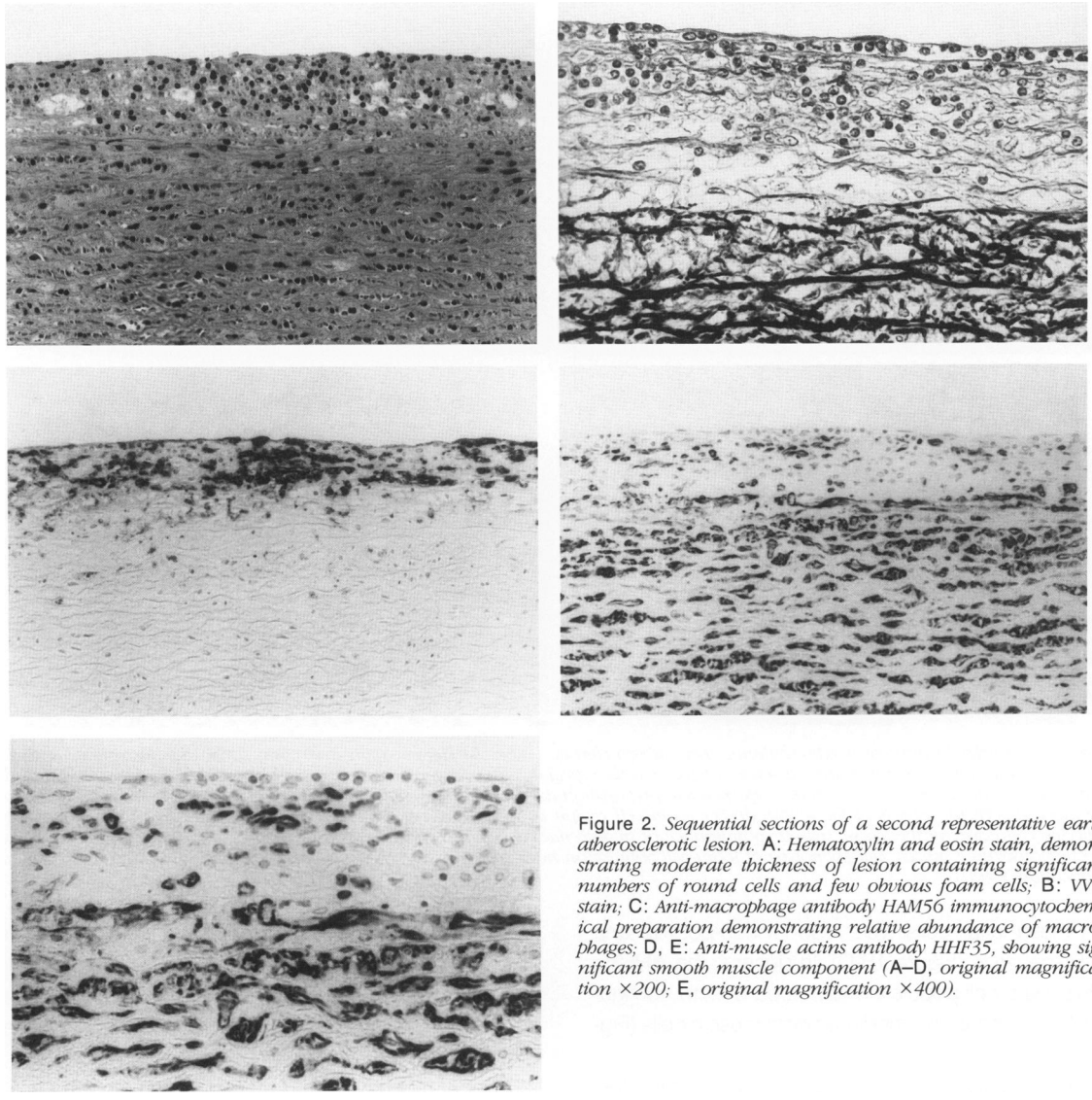

Figure 2. *Sequential sections of a second representative early atherosclerotic lesion.* A: *Hematoxylin and eosin stain, demonstrating moderate thickness of lesion containing significant numbers of round cells and few obvious foam cells;* B: *VVG stain;* C: *Anti-macrophage antibody HAM56 immunocytochemical preparation demonstrating relative abundance of macrophages;* D, E: *Anti-muscle actins antibody HHF35, showing significant smooth muscle component (*A–D, *original magnification ×200;* E, *original magnification ×400).*

demonstrated an intact endothelium over these lesions in virtually all cases (Figure 3A). Using the same reagents, vascularization of the underlying aortic media could be demonstrated in several specimens. Where this occurred, the vasa vasorum were often accompanied by accumulations of anti-CD45-positive lymphocytes and HAM56-positive macrophages (Figure 4).

Anti-desmin antibodies identified only a subset of the smooth muscle cells that stained positively with antibody HHF35. In general, desmin-positive smooth muscle cells were restricted to the outer half of the media, although there was considerable variation. In rare cases, desmin-positive smooth muscle cells were present within the lesions (Figure 3B). Furthermore, rare HHF35-positive smooth muscle cells coexpressed cytokeratin 8, as determined by reactivity with antibodies MAK6 and 35βH11 (Figure 3F).

Discussion

Lesions in Adolescents and Young Adults

In the present study, we have analyzed the cellular composition of early human aortic lesions that had gross morphologic features characteristic of fatty streaks observed in individuals between the ages of 15 and 34. The histologic appearance of these lesions belied their somewhat uniform gross appearance, since many had features generally associated with more advanced lesions, such

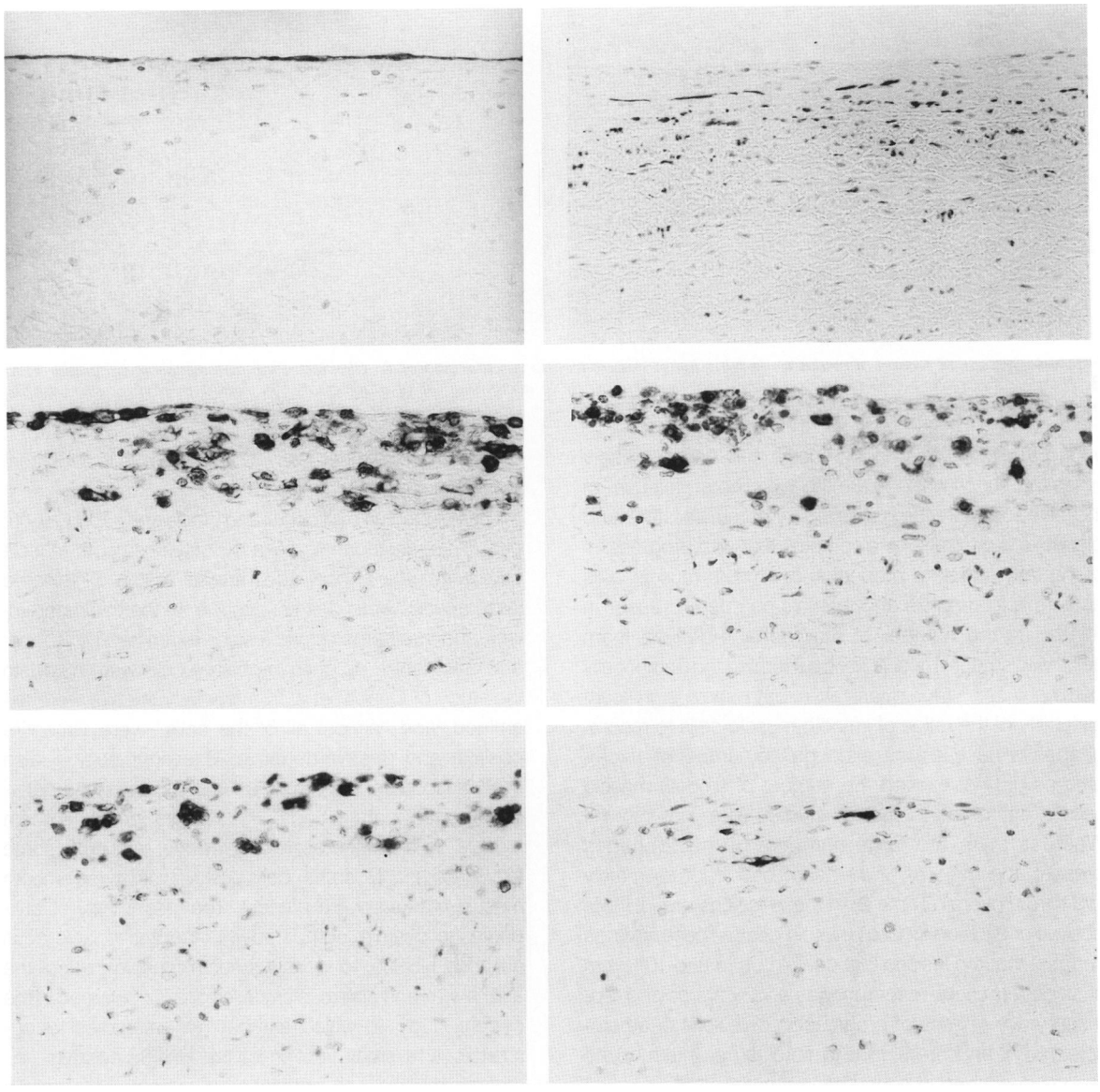

Figure 3. *Sequential sections of representative lesion.* **A**: *Ulex lectin immunocytochemical preparation demonstrating intact endothelium covering lesion;* **B**: *Anti-desmin immunocytochemical preparation, showing poor expression of this muscle protein by smooth muscle cells of aorta and lesion;* **C**: *Anti-CD45 immunocytochemical preparation demonstrating lymphocytic composition of the lesion;* **D, E**: *Anti-CD45RO (D, antibody UCHL-1; E, antibody OPD4), immunocytochemical preparation demonstrating T-cell nature of vast majority of cells;* **F**: *Anti-cytokeratin 8 immunocytochemical preparation showing presence of scattered positive cells, probably representing subset of smooth muscle cells (***A, C–F,** *original magnification ×400;* **B,** *original magnification ×200).*

as significant numbers of smooth muscle cells. Although all appeared to be fatty streaks grossly, they might be better characterized as "fibrofatty" lesions, or advanced fatty streaks. As described by Stary in a series of papers, fatty streaks, or type II lesions, at least in the coronary arteries, where best studied, appear to arise in an artery wall already characterized by diffuse or eccentric intimal thickening composed predominantly of smooth muscle cells. Given the limited number of lesions studied here, it is difficult to determine with certainty where in the proposed sequence of events from fatty streak to fibrofatty (type III) lesion, the specimens in this study would fit.[7–9]

Using a panel of antibodies, we identified all of the principal participant cell types in these lesions. This antibody panel is similar to that which we used in our previous study of fibrous plaques.[1] We have added newly available antibodies to study the subsets of lymphocytes in the lesions as well.

The Predominant Cell is Smooth Muscle

One of the major findings of this study is the elucidation that the predominant cell component of these lesions in

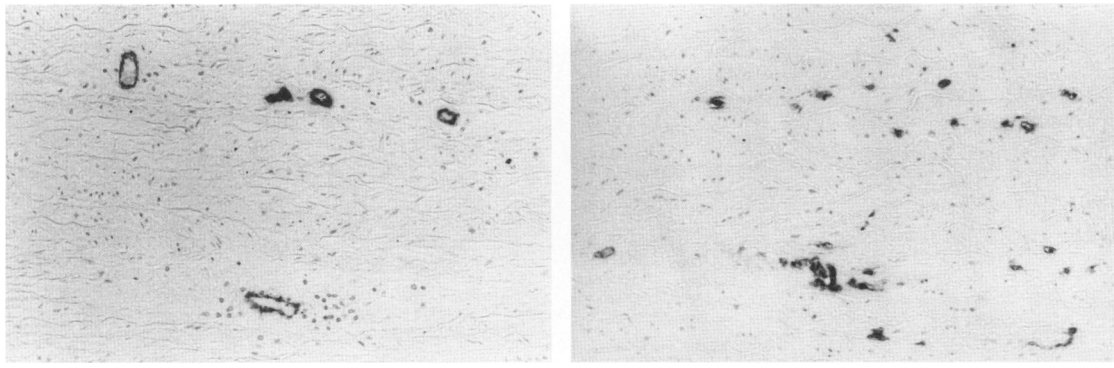

Figure 4. *Sequential section of media underlying lesion demonstrating vascularization.* **A**: *Ulex lectin immunocytochemical preparation;* **B**: *HAM56 anti-macrophage immunocytochemical preparation. Note accumulation of macrophages in media in regions surrounding vascularization (***A, B,** *original magnification ×200).*

young adults is the smooth muscle cell. Although Stary notes that macrophages are the "first line of defense" of the intima, and are the first cells to accumulate, store, and digest lipid droplets, he also notes that with progression of the fatty streak, a proportion of smooth muscle cells acquire lipid droplets and that this number increases in relation to the number of macrophage-derived foam cells, with smooth muscle cells becoming the numerically dominant cell.[8] Our quantitative data show significant variability of the ratios of smooth muscle cells to macrophages in these lesions, although the number of macrophages did not exceed the number of smooth muscle cells in any of the 27 lesions studied (data not shown). Whereas there are large numbers of macrophages present, the majority of the foam cells (approximately two-thirds, overall; Table 2) in these lesions are HHF35-positive, indicating smooth muscle origin. These findings suggest that the lesions that contain up to 8 to 10 layers of smooth muscle, macrophages and lymphocytes represent early phases of progression of lesions of atherosclerosis,[10] and are similar to fibrofatty or intermediate lesions described in nonhuman primates.[11–14]

The results regarding cell composition are at some variance with some previously reported immunocytochemical studies of human fatty streaks, in which the macrophage was reported as the dominant component.[15–19] However, most, if not all, of these previous reports are not directly comparable to the study reported here. The number of lesions examined here, 27, is greater than those reported by previous investigators.[15–18] The studies of Aqel et al,[15] Klurfeld,[16] and Munro et al,[17] were immunocytochemical investigations performed on frozen sections of lesions. Our study, like that of Roessner et al,[18] employed fixed, paraffin-embedded material, that affords better preservation of tissue architecture and cellular details. Thus, our studies permit more precise determination of the fraction of each cell type identified with each of the various antibodies. In one recent study employing similar materials and antibodies, predominance of smooth muscle cell-derived foam cells in lesions from young adults was also found.[20]

Earlier studies by Kocher and Gabbiani[21] and Osborn et al,[22] suggested that only a minority of smooth muscle cells within the normal adult arterial media of selected large arteries were desmin-positive. In the thickened intima and in atherosclerotic lesions examined by Kocher and Gabbiani,[21] no desmin-positive cells were noted. In the study of Osborn et al,[22] a few fatty streaks were examined, and "almost all of the cells" were vimentin-positive and desmin-negative. Roessner et al,[18] also used antibodies to desmin, as well as an uncharacterized monoclonal antibody, HM-19/2, to identify smooth muscle cells in atherosclerotic lesions, including some fatty streaks; only "some" cells positive with these smooth muscle markers were present. The insensitivity of antibodies to desmin, compared with the anti-muscle actin antibody HHF35, to identify smooth muscle cells of the arterial wall and atherosclerotic lesions is confirmed in the current study, in which anti-desmin antibodies recognized only a small fraction of the HHF35-positive, i.e., smooth muscle cell population. Consequently, only a small fraction of the smooth muscle cell population can be identified with antibodies to desmin, and studies such as that of Roessner et al,[18] probably underestimated the number of smooth muscle cells in the lesions. That a subset of the smooth muscle cell population of the lesions express desmin provides further evidence of phenotypic heterogeneity within this cell population. Our results also demonstrate that a small subset of smooth muscle cells within these lesions apparently coexpress cytokeratin 8, in keeping with previously reported studies on smooth muscle cells of the myometrium and atherosclerotic plaque.[22,23]

Most Round Cells are Macrophages

The use of different antimacrophage antibodies may also account for some of the differences in the previously re-

ported data and our observations. In our study, the location of the monocyte-macrophage–derived foam cells was different from that of their smooth muscle-derived counterparts. Specifically, the HAM56-positive (i.e., macrophage-derived) foam cells were found predominantly in the upper and middle layers of the lesions, covered by an HHF35-positive (i.e., smooth muscle) "cap." In contrast, the HHF35-positive foam cells were present predominantly in the deeper layers of the lesions, contiguous with the underlying media. Munro and colleagues[17] have demonstrated that approximately 90% of the foam cells in lesions they examined reacted with the anti-macrophage antibody RFD-2, and that the anti-tissue macrophage antibody RFD-7 reacted with 25–85% of the foam cells in the fatty streaks. In these cases, foam cells were generally described as located in a predominantly subendothelial distribution, although these authors also found RFD-7-negative foam cells in the deeper layers of the lesions. It is not clear whether the antibodies used by Munro et al,[17] crossreact with smooth muscle-derived foam cells since no data were presented concerning possible crossreactivity.

Both Earlier and Later Lesions Require Study

A major reason for the discrepancy between our results and those reported earlier may be the difference in stage of development of lesions studied, despite similar nomenclature and possibly even gross appearances. For example, it is not clear that Aqel et al,[15] examined lesions comparable to those we investigated; only four specimens designated "uncomplicated atherosclerotic plaque[s]" from two individuals (aged 34 and 45) were examined in the study. These could have represented early fibrous plaques rather than fatty streaks, based on the limited gross description and the age of the patients. All the lesions examined by Munro et al[17] were obtained from a different patient population than that examined in the current study. The specimens of lesions in their study were obtained from proximal aortic wall in patients undergoing aortocoronary bypass grafts, and by definition, were advanced, occlusive lesions. Although the age of the patient population was not reported, the fatty streaks examined by Munro et al[17] were probably derived from an elderly population, and may not correspond to the lesions observed in the young individuals examined in our study. Based on our previous observations in Watanabe and fat-fed rabbits[25–27] and fat-fed monkeys,[11–14] in which the early accumulation of monocyte-macrophage cells is accompanied by, and eventually exceeded by, infiltration and proliferation of smooth muscle cells, the present observations are consistent with the interpretation that the lesions examined here probably represent "advanced fatty streaks" or fatty streaks in transition to becoming fibrous plaques.

The results of this study contrast sharply with the cell composition observed in more advanced human lesions, as published previously using similar antibodies and techniques.[1,28,29] In more advanced lesions, there is greater heterogeneity with respect to cell composition, with more variation from region-to-region within individual lesions. It will be interesting to apply these techniques and antibodies to examine lesions obtained from a younger population as well as those temporally intermediate between these advanced fatty streaks and the more advanced lesions previously examined. Such studies may shed further light on the biology of the advancing human lesion, and the relationship between the early fatty streaks and the more advanced, complex lesions.

Note Added in Proof

Since submission of this manuscript, a study of Poppema et al[30] suggests that the reactivity of antibody OPD4 is restricted to T lymphocytes and does not include monocytes.

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