Human Atherosclerosis

II. Immunocytochemical Analysis of the Cellular Composition of Human Atherosclerotic Lesions

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The authors have performed immunocytochemical investigations of the distribution of various cell types in human atherosclerotic plaques using monoclonal antibodies specific to smooth muscle cells (CGA7 [Gown et al, J Cell Biol 1985, 100:807–813] and HHF35 [Tsukada et al, Am J Pathol (In press)]); lymphocytes (T200 antigen); endothelial cells (Factor VIII and the Ulex europeus agglutinin); and macrophages, the latter with a new macrophage-specific antibody HAM56. All studies were performed on methanol–Carnoy's-fixed, paraffin-embedded tissues. In areas of grossly normal aorta, significant numbers of macrophages were noted within areas of diffuse intimal thickening. The cellular composition of the following three types of raised lesions were analyzed: 1) fibro-fatty lesions, which, despite their gross appearance, consistent with fibrous plaques, were composed almost exclusively of macrophages and lymphocytes and almost devoid of smooth muscle cells; 2) fibrous plaques, which were predominantly composed of smooth muscle cells displaying considerable morphologic heterogeneity and an admixture of blood-borne cells; 3) advanced plaques, which were characterized by complex layers of smooth muscle cells and macrophages with considerable variation from region to region. Also noted were foci of medial and even intimal vascularization subjacent to the more advanced plaques. These studies demonstrate the application of monoclonal antibody technology to the study of the cellular composition of human atherosclerotic lesions. (Am J Pathol 1986, 125:191–207)

SEVERAL types of cells have been recognized in the lesions of human atherosclerosis. It is generally agreed that the major cell found within the advanced lesion of human atherosclerosis, the fibrous plaque, is the smooth muscle cell, presumably derived at some time during lesion development from medial smooth muscle cells.[1] Nonetheless, histochemical and electron-microscopic studies have recently suggested that in many lesions there is a significant component of monocytes and/or macrophages, which may depend, in part, upon the stage of the process examined (eg, fibrous plaque versus fatty streak or foam cell lesions).[2,3]

Positive identification of smooth muscle cells has traditionally been made with the use of morphologic criteria alone, with or without ultrastructural corroboration.[4] Nonetheless, these studies make certain assumptions about the range of morphologic features that may or may not be expressed by smooth muscle cells during the course of plaque development. It would be valuable to have a marker for smooth muscle cells that is independent of these strictly morphologic variables. Identification of monocytes and macrophages has been more difficult; in addition to morphologic identification,[2,5] histochemical markers such as nonspecific esterase,[6,7] peroxidase,[6,8] and acid lipase[8–10] and Fc and C3 receptor activity[3,11,12] have been used, but almost exclusively in the study of nonhuman lesions. Furthermore, many of these latter markers were used in disrupted tissue specimens, and most are not amenable for use in fixed, embedded tissue sections where morphologic preservation is optimal. Consequently, despite the availability of these methods, it is still not possible

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to positively identify all of the smooth muscle cells or monocytes and macrophages and simultaneously describe their distribution in human atherosclerotic lesions. It is particularly difficult to determine cell type after cells become heavily lipid-laden and take on the appearance of foam cells. Evidence has been presented that many foam cells in human lesions are of monocyte-macrophage origin,[3] although it is possible that some lesion foam cells are actually of smooth muscle cell origin. Monoclonal antibodies have been demonstrated to be useful in the positive identification of macrophages in rabbit[13] and human[14-17] lesions. To completely address this issue, however, one needs the simultaneous use of markers specific for smooth muscle cells and macrophages. It would also be important to identify the other cell types present in the artery wall and in atherosclerotic lesions, ie, endothelial cells, lymphocytes, and other blood-borne cells. Aqel et al[16] have recently studied human atherosclerotic lesions with monoclonal antibodies to smooth muscle cells and macrophages, although the work by necessity has been performed on frozen tissue sections, where morphologic preservation is suboptimal. We have addressed the problems of tissue preservation as well as cell identification by developing cell-specific monoclonal antibodies that can be used in methanol-Carnoy's-fixed, paraffin-embedded sections of human atherosclerotic lesions, yielding simultaneous information about the morphology and distribution of the various cell components of these lesions.

Materials and Methods

Sources of Antibodies

The sources and specificities of all antibodies used in this study are summarized in Table 1.

Briefly, antibody 43βE8 is a monoclonal anti-vimentin antibody, the isolation and characterization of which has been previously described.[18,19] Antibody CGA7 is a monoclonal antibody that reacts with smooth-muscle-specific alpha and gamma actin isotypes; data regarding this antibody and its specificity have been published elsewhere.[20] Antibody HHF35 is a monoclonal anti-actin antibody that recognizes actin isotypes (alpha and gamma) common to all muscle cells, including smooth muscle cells; documentation of its specificity has been published elsewhere.[21,22] These latter two antibodies do not react with fibroblasts, endothelial cells, lymphocytes, monocytes or macrophages. Moncolonal antibodies to Factor VIII-related antigen were obtained from Cappel Laboratories (Catalog No. 0201-3207); Factor VIII-related antigen has been well characterized as an endothelial-cell-specific marker in tissue sections.[23,24] *Ulex europaeus* agglutinin (UEA) (Vector Laboratories, Burlingame, Calif) and polyclonal antibodies to UEA (Vector Laboratories) were used sequentially as additional endothelial markers.[25] Monoclonal antibodies to the T200 (pan-lymphoid) antigen were obtained from Dako (Catalog No. M-701); T200 is present on T and B lymphocytes, monocytes, and at least some macrophages,[26] and this particular antibody has been demonstrated to identify the antigen in fixed, embedded sections.[27]

Generation of Anti-Macrophage Monoclonal Antibody

An anti-macrophage monoclonal antibody, designated HAM56, was developed specifically for use in these studies. Human alveolar macrophages were obtained from healthy adult volunteers with the use of lung lavage via bronchoscopy. Cells thus obtained were centrifuged at 800 rpm for 5 minutes and aliquots either injected intraperitoneally into BALB/c mice with complete Freund's adjuvant or frozen for future injections. Following brief sonication, intraperitoneal injections of approximately 1×10^6 cells were subsequently given at Week 2 and Week 3. The Köhler-Milstein procedure, modified as described previously[18] was used; clones were screened on methanol-Carnoy's-fixed, paraffin-embedded sections of human lung and an advanced femoral atherosclerotic plaque. Several clones designated HAM56, HAM101, HAM118 were thus obtained; reliable reactivity on fixed, embedded human tissues was best with antibody HAM56. Ouchterlony double diffusion immunoprecipitation methods revealed that HAM56 was of the IgM subclass.

Supernatant fluids and mouse ascites fluids derived from corresponding clones were further screened on smears derived from Ficoll-Hypaque gradient-separated peripheral blood lymphocytes, prepared according to standard methods.[28] The reactivity of the antibody on isolated blood cells was directly compared with that of the following commercially available monoclonal antibodies: anti-macrophage antibodies OKM1 and OKM5 (Ortho Diagnostic Systems); anti-macrophage antibody Leu-M3 (Becton Dickinson); anti-pan-B-cell antibody OKB7 (Ortho Diagnostic Systems); anti-pan-T cell antibody OKT11 (Ortho Diagnostic Systems). Anti-Ia antibody 7.2[29] was kindly given by Dr. Paul Martin, Fred Hutchinson Cancer Research Center, Seattle, Washington. Double immunofluorescence studies were performed by simultaneous incubation of the isolated cells with working dilutions of two antibodies, followed by an intervening wash in phosphate-buffered saline (PBS), followed by simultaneous incubation with 1:20 dilutions in PBS of rhodamine-conjugated goat anti-

Table 1—Monoclonal Antibodies Used in Immunocytochemical Analysis

Designation	Specificity	Cells identified	Reference/source	Working dilution
43βE8	Vimentin	Fibroblasts Smooth muscle cells Macrophages Endothelial cells	Reference 18	1:1000*
CGA7	SMC actin	Smooth muscle cells	Reference 20	1:1000*
HHF35	Muscle actins	Smooth muscle cells	Reference 21	1:8000*
–	Factor VIII-Rag	Endothelial cells	Cappel Laboratories	1:500*
HAM56	Undetermined	Macrophages Endothelial cells†	This paper	1:2000*
M701	T200 Antigen	Lymphocytes Monocytes Macrophages†	Dako Laboratories	1:10‡
–	UEA	Endothelial cells	Vector Laboratories	1:500§

* As ascites fluids.
† Variable reactivity (see text).
‡ As supernatant fluid.
§ As polyclonal antibody diluted as supplied by manufacturer.

mouse IgM and fluorescein-conjugated goat anti-mouse IgG (Cappel). (All of the commercially available macrophage, lymphocyte, and Ia monoclonal antibodies are murine IgGs.) After a final wash in PBS, the slides were viewed on a Leitz fluorescence microscope equipped with appropriate barrier filters. Finally, ascites fluids were used in standard immunoblot experiments on various cell extracts after polyacrylamide gel electrophoresis.[30]

Cell Culture

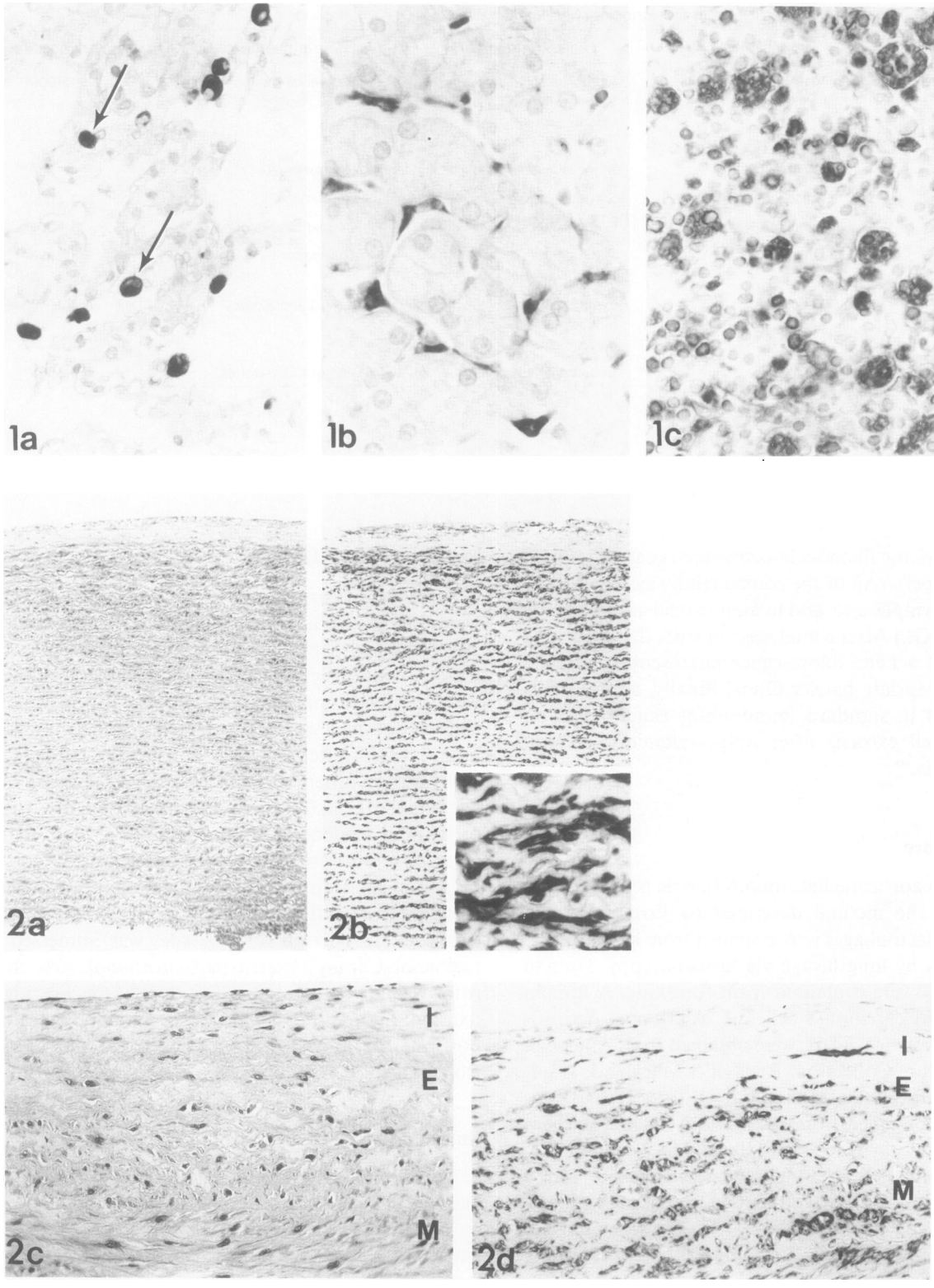
Human aortic medial smooth muscle cells were prepared by the method described by Ross.[31] Human alveolar macrophages were obtained from healthy adult volunteers by lung lavage via bronchoscopy. Human fibroblasts were explanted from foreskins. A human epidermoid carcinoma cell line, A431, and erythroleukemia cell line, K562, were obtained from American Type Culture Collection, Rockville, Maryland. A human hepatocellular carcinoma cell line, Hep3B, was originally obtained from Dr. David Aden and Barbara Knowles, Wistar Institute, Philadelphia, Pennsylvania. A melanoma cell line, Mel-1, was established from a portion of an axillary lymph node containing pigmented melanoma metastasis.[32] Two variants of a normal B-lymphoblastoid cell, 6.1.6 and 8.1.6,[33] were kindly given by Dr. Donald Pious, University of Washington. The cells were cultured in RPMI-1640 medium (GIBCO Laboratories) supplemented with 15% fetal calf serum, L-glutamine (1 mM), sodium pyruvate (1 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml, GIBCO).

Procurement of Tissue

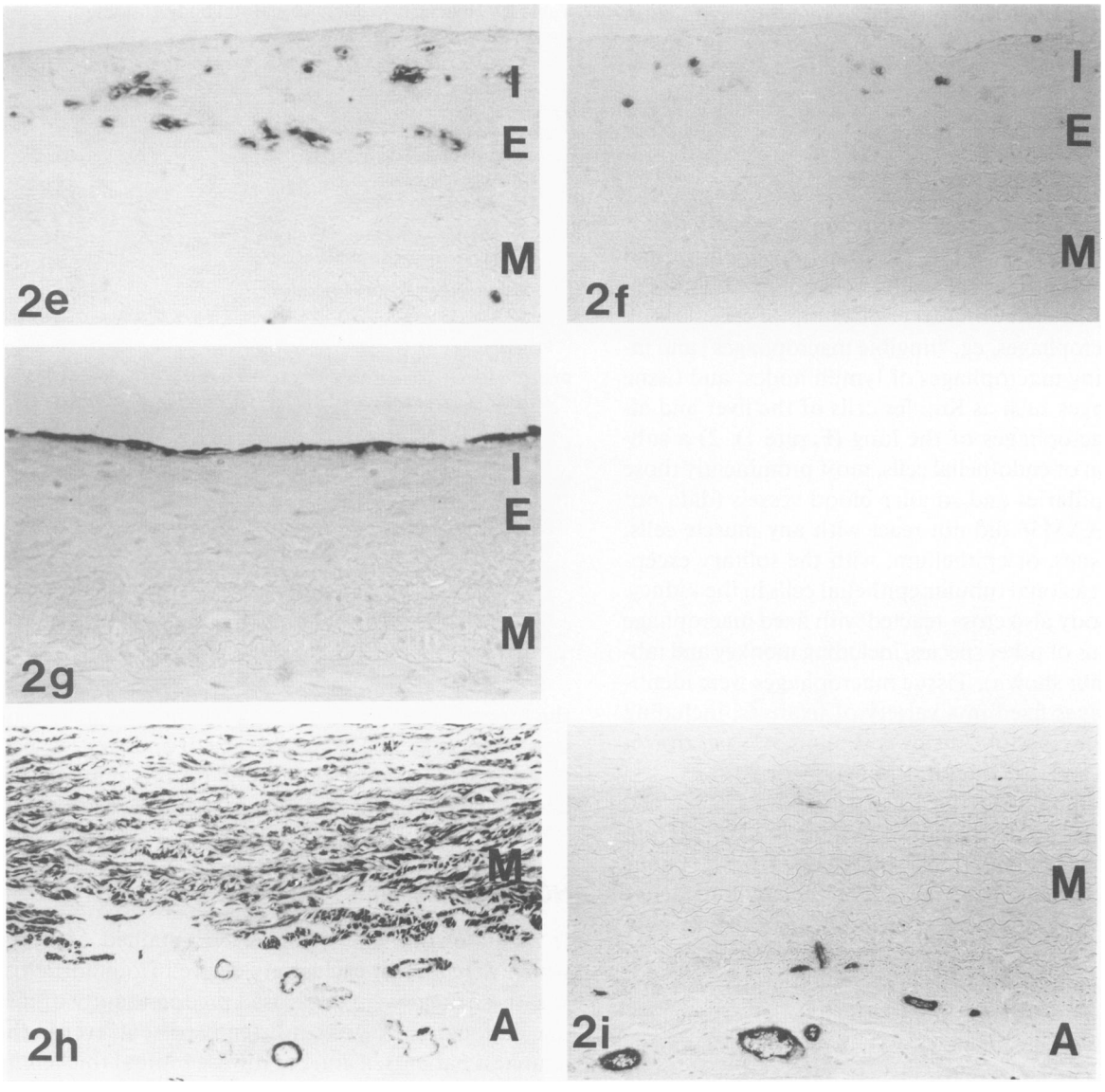
Segments of carotid and femoral arteries and aorta were obtained from the Surgical Pathology and Autopsy Pathology services of University Hospital, Seattle. Carotid endarterectomy and femoral bypass specimens were obtained as previously described.[4] Autopsy material was obtained within 12 hours of death (range, 4–12 hours). In the latter material, which was almost exclusively aortas, atherosclerotic lesions were selected that were small (0.2–2.0 cm in diameter), raised, gray, with or without evidence of an atheromatous base. Fatty streaks or dots were not included in this study. Patients ranged in age from 34 to 58 years; a total of 96 specimens from 68 individuals (42 men, 26 women) were examined. A portion of all tissues was immersed in methanol-Carnoy's fixative (60% methanol, 30% chloroform, and 10% glacial acetic acid). After overnight fixation, tissues were embedded in paraffin. A second portion was snap-frozen in liquid-nitrogen-cooled isopentane.

Immunocytochemistry

The avidin-biotin complex immunoperoxidase system with nickel chloride color modification as previously described[19] was employed both on acetone-fixed, frozen sections and deparaffinized, methanol-Carnoy's-fixed tissue sections. In the case of the latter tissue, with intervening washes in PBS, sections were deparaffinized and rehydrated to PBS. For all tissue, primary antibodies were applied at dilutions as indicated in Table 1; localization with the antibody to UEA required prior incubation of the deparaffinized sections with UEA



Figures 1–7 all represent methanol–Carnoy's-fixed, paraffin-embedded sections reacted with indicated antibodies with localization via the avidin–biotin immunoperoxidase method. Abbreviations for all figures: *M*, media; *E*, internal elastic lamina; *I*, intima; *A*, adventitia. **Figure 1**—Monkey lung section reacted with antibody HAM56; only alveolar macrophages are positive (*arrows*). (Original magnification, ×400) **b**—Human liver section reacted with antibody HAM56, only the Kupffer cell population is positive. (Original magnification, ×500) **c**—Reactive human lymph node section reacted with antibody HAM56; note positive macrophages with background lymphocytes negative. (Original magnification, ×300) **Figure 2**—Nonlesional aorta. **a**—H&E-stained preparation; note mild diffuse intimal thickening. (Original magnification, ×80) **b**—Serial section reacted with anti-muscle cell antibody HHF35; note uniform immunoreactivity of medial smooth muscle cells. (Original magnification, ×80) **Inset**—Higher magnification of medial smooth



muscle cell reacted with antibody HHF35. (Original magnification, ×500) **c–g**—Serial sections of intimal portion of this specimen reacted with (**c**) H&E stain, **d** anti-muscle cell antibody HHF35, **e** anti-macrophage antibody HAM56, (**f**) anti-T200 antibody, (**g**) anti-Factor VIII antibody. Note the presence of smooth muscle cells, scattered macrophages, rare lymphocytes, and preservation of overlying endothelial cells. The media does not contain macrophages or lymphocytes. (Original magnification, ×160) **h** and **I**—Sections of deep media and adventitia. **h**—Anti-muscle cell antibody HHF35. **I**—Anti-Factor VIII. Note positive smooth muscle cells in deep media and adventital vessel walls; Factor VIII antibodies highlight the adventitial vessel endothelium. No penetration of these vessels into the media is noted. Adventitial fibroblasts do not react with antibody HHF35. (**h** and **I**, original magnification, ×156)

(Vector Laboratories, Burlingame, Calif; 1:100 dilution of 2 mg/ml solution in PBS). With intervening washes in PBS, sections for all antibody localization studies were serially incubated in 1:500 dilutions in PBS of biotinylated goat anti-mouse, anti-rabbit, or anti-goat IgG, corresponding to a dilution of 0.003 mg/ml (Vector), 30 minutes; avidin–biotinylated horseradish peroxidase complex (Vector) at a 1:1 ratio, as supplied by the manufacturer; a 0.1 M solution of 3,3′-diaminobenzidine (DAB, Sigma Chemicals, St. Louis, Mo) in 0.05 M Tris buffer, pH 7.6 (10 minutes) to which had been added 0.75 ml of a 3% H_2O_2 and 1.0 ml of an 8% NiCl solution in PBS. For the vimentin antibody only pretreatment of the deparaffinized sections with a 0.1 M solution of Pronase (70,000 U/g; Calbiochem-Behring, San Diego, Calif) was required. Sections were counterstained with methyl green and dehydrated in sequential alcohols, and then mounting media and coverslips were applied. For double-labeling immunoenzyme experiments, sequential avidin–biotin immunoperoxidase procedures were used, with the first antibody (HAM56) localization enhanced with the addition of nickel chloride as above to the diaminobenzidine to yield a black reaction product, and the second procedure unmodified, to

yield a brown reaction product. This double-labeling procedure has been described by Hsu and Soban.[34]

Results

Specificity of Antibody HAM56

When tested on a panel of frozen, acetone-fixed or methanol-Carnoy's-fixed, paraffin-embedded normal human tissues, identical results were obtained. HAM56 exclusively reacted with two populations of cells: 1) fixed tissue macrophages, eg, "tingible macrophages" and interdigitating macrophages of lymph nodes, and tissue macrophages such as Kupffer cells of the liver and alveolar macrophages of the lung (Figure 1); 2) a subpopulation of endothelial cells, most prominently those of the capillaries and smaller blood vessels (data not shown). HAM56 did not react with any muscle cells, neural tissues, or epithelium, with the solitary exception of occasional tubular epithelial cells in the kidney. The antibody also cross-reacted with fixed macrophage populations of other species, including monkey and rabbit (data not shown). Tissue macrophages were identified in tissues fixed in a variety of fixatives, including Carnoy's, methanol-Carnoy's, Bouin's, 4% paraformaldehyde, and 10% neutral buffered formalin.

Table 2 summarizes the reactivity of HAM56 in blood and cultured human cell lines. When tested on peripheral blood cell populations isolated by Ficoll-Hypaque density gradient, HAM56 was completely nonreactive on platelets, granulocytes, or red blood cells. In double immunofluorescence studies on isolated mononuclear cells, none of the OKT11- or OKB7-positive T and B lymphocytes were positive with HAM56. Of the Leu-M3-positive cells, 95% were HAM56-positive. Corresponding percentages for OKM1, OKM5, and anti-Ia were 96, 90, and 86. Thus, on peripheral blood cells HAM56 reacted exclusively with a large subset of the monocyte/macrophage population, as defined by commercially available monoclonal antibodies. On cultured cells, HAM56 was positive with 100% of primary cultures of alveolar macrophages, but completely negative on three mesenchymal cells (human smooth muscle cells, fibroblasts, and Mel-1), two epithelial cell lines (A431 and Hep3B), and three lymphocyte cell lines (K562, 6.1.6, and 8.1.6).

Immunoblotting experiments failed to reveal any positive bands in extracts of alveolar macrophages or any other cell population.

Table 2—Reactivity of Monoclonal Antibody HAM56 on Human Blood and Cultured Cells

	% Positive cells
Peripheral blood cells	
Monocytes	
OKM1-positive cells	96
OKM5-positive cells	90
Leu-M3-positive cells	95
T-lymphocytes—OKT11-positive cells	0
B-lymphocytes—OKB7-positive cells	0
Ia-positive cells—7.2-positive cells	86
Granulocytes	0
Platelets	0
Primary human cultured cells	
Alveolar macrophages	100
Smooth muscle cells	0
Fibroblasts	0
Human cell lines	
A431 (epidermoid carcinoma)	0
Hep3B (hepatocellular carcinoma)	0
Mel-1 (melanoma)	0
6.1.6 (transformed B lymphocyte)	0
8.1.6 (transformed B lymphocyte)	0
K562 (erythroleukemia)	0

Immunocytochemical Analyses

Identical results were obtained on the fixed, embedded material and the acetone-fixed, frozen sections. Because the former material afforded vastly superior histologic preservation as well as an absence of endogenous peroxidase activity, only the results on this material are described. The major findings are summaried in Table 3.

Nonlesional Areas

In hematoxylin and eosin (H&E)-stained sections, which were almost exclusively derived from aortic tissue, these areas were composed predominantly of media, although there was consistently present, even in the youngest patients, a zone of diffuse intimal thickening (Figure 2a and c).

The medial smooth muscle cells were clearly delineated by their uniform immunoreactivity with antibodies CGA7 and HHF35 (Figure 2b). Myointimal cells within the areas of diffuse intimal thickening were also noted to be CGA7-positive and HHF35-positive, albeit with slightly different morphologic features (Figure 2d). Scattered throughout this zone of diffuse intimal thickening were small numbers of HAM56-positive macrophages and, often, other anti-T200-positive blood-borne cells (Figure 2e and f). The ratio of macrophages to smooth muscle cells in these lesions can be gleaned from double-labeling studies as seen in Figure 8a. HAM56-positive cells were never seen within the subjacent (normal) media. Factor VIII antibodies, as well as those to UEA, were useful in demonstrating the preserved endothelial surface covering the intima, as well as adventitial vessels (Figure 2g and i). As expected, adventitial fibroblasts were not positive with either HHF35 or

Table 3—Summary of Antibody Reactivities of Human Atherosclerotic Lesions

	HHF35 CGA7 (smooth muscle cells)	HAM56 anti-T200 (monocytes/lymphocytes)	Anti-Factor VIII UEA (endothelial cells)
Normal wall	Almost all cells positive	Rare scattered cells positive	Surface endothelium present
Fibrofatty lesion	Rare cells positive	Almost all cells positive	Surface endothelium variably present
Fibrous plaque	Variable numbers of positive cells present; morphologic heterogeneity present	Variable numbers of positive cells present	Surface endothelium variably present
Advanced plaque	Variable numbers of positive cells present; morphologic heterogeneity present	Variable numbers of positive cells present	Surface endothelium variably present; patchy vascularization of media and plaque noted

CGA7 (Figure 2h), but were positive with the anti-vimentin antibody 43βE8 (Figure 7b).

Fibro-fatty Lesions

On H&E-stained sections, fibro-fatty lesions were characterized by a loose connective tissue matrix, containing rather evenly distributed cells, the nature of which was indeterminate by histologic study alone. Rare foam cells were noted. These lesions generally measured less than 0.20 cm in size (Figure 3a). In these lesions, overlying endothelium was variably intact (data not shown). Only rare cells within the lesion were HHF35-positive or CGA7-positive (Figure 3b). The overwhelming majority of these cells were, in fact, HAM56-positive (Figure 3c); a variable number were anti-T200-positive as well (Figure 3d), suggesting that these represented monocyte/macrophage lesions with only a minimal smooth muscle cell component. The underlying medial smooth muscle cells were uniformly HHF35-positive and CGA7-positive (Figure 3b). No HAM56-positive cells were seen within the subjacent media (Figure 3c).

Fibrous Plaques

On H&E-stained sections, fibrous plaques contained a classic cellular fibrous cap overlying a rather acellular core which either lacked or contained atheromatous debris. Foam cells were variably present. These lesions varied widely in size, ranging from 0.1 to 2 cm (Figure 4a).

The predominant cell type of these lesions was the HHF35-positive and CGA7-positive smooth muscle cell (Figure 4b). In general, the immunocytochemical preparations using the muscle-specific antibodies revealed far greater numbers of smooth muscle cells in the fibrous caps than were suggested by H&E-stained slides.

These lesions showed marked heterogeneity in the distribution as well as the morphologic features of the smooth muscle cell population. For example, HHF35-positive and CGA7-positive smooth muscle cells were present in a nonrandom fashion throughout the fibrous cap. Cells with the same "immunophenotype" were present in clusters deep to atheromatous areas directly above the media, as well as some clusters in a subendothelial distribution, apart from other smooth muscle cells of the cap. By their immunocytochemical staining patterns, at least two distinct "phenotypes" of HHF35-positive and CGA7-positive smooth muscle cells were apparent (Figure 4e), and each was found in a fairly consistent locus within the lesions. The first were pancake-shaped cells, corresponding to the extremely long, flat cells described in our previous ultrastructural investigations[4] (Figure 4f). These cells were invariably found only in the fibrous cap portion of the lesion; many zones which would be dismissed as acellular on the basis of standard H&E preparations were found to contain suprising numbers of these cells. Indeed, one suspects that the numbers of these cells, even as ascertained by immunocytochemical preparations, may have been underestimated because of artefactual loss of some of them, which were often compressed to the edge of their lacunae, during fixation and processing.[4] The second population of smooth muscle cell was identified by their dense immunostaining at the cell periphery with antibody HHF35 (Figure 4g). They were more plump than the pancake-shaped cells and did not have the extremely long profiles of the latter. These latter cells generally populated the regions of intimal thickening subjacent to the pancake-shaped cells. In the more advanced lesions, at least one other morphologic variant of smooth muscle cells was also noted (see below).

A significant subpopulation of macrophages and lymphocytes was consistently demonstrated, as shown

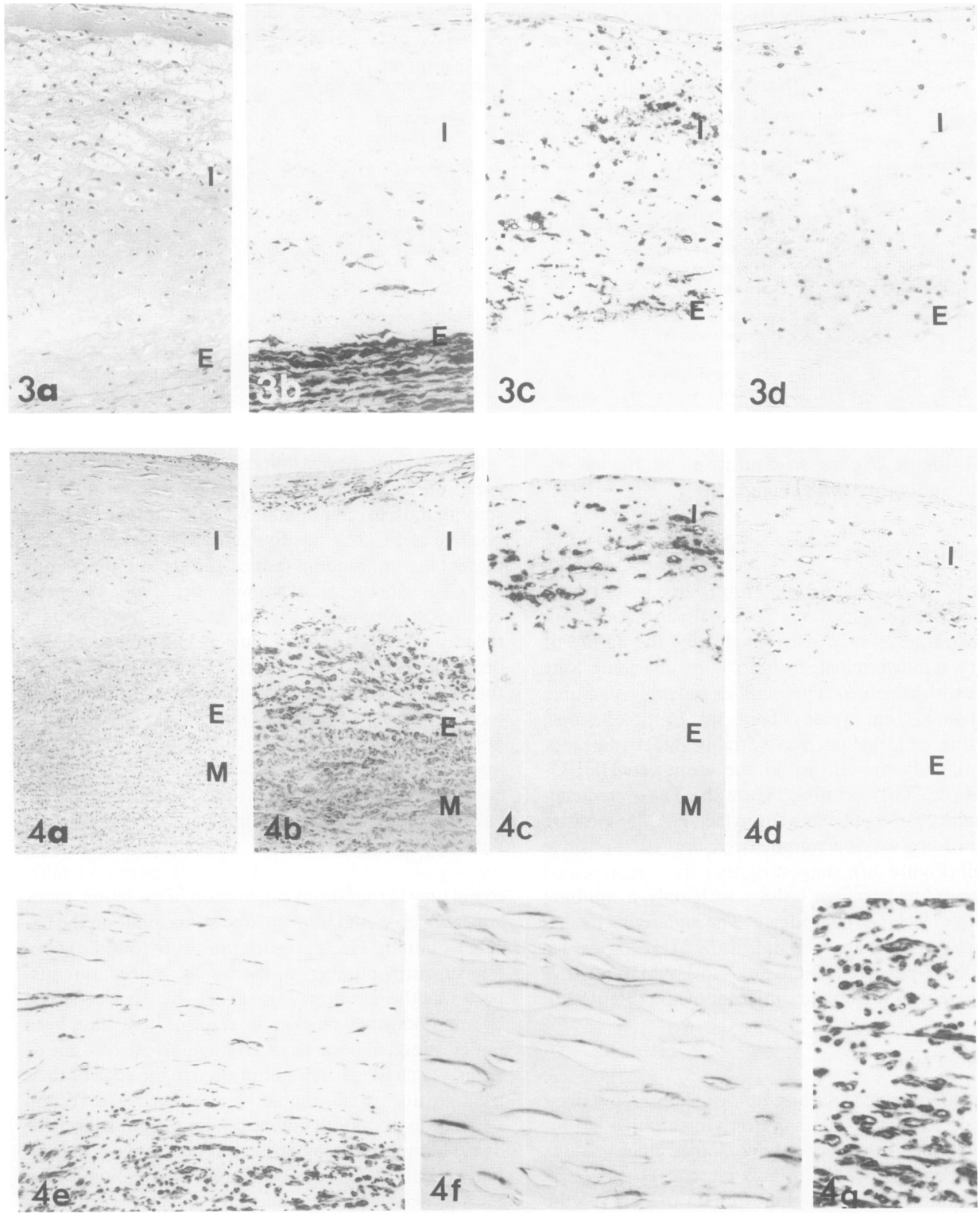


Figure 3—Fibro-fatty lesion from aorta. Serial sections reacted with H&E (**a**), anti-muscle cell antibody HHF35 (**b**), anti-macrophage antibody HAM56 (**c**), and anti-T200 antibody (**d**). Note the large numbers of macrophages and relative dearth of smooth muscle cells in the lesion. (Original magnification, ×240) **Figure 4**—Fibrous plaque from aorta. **a–d**—Serial sections reacted with H&E (**a**), anti-muscle cell antibody HHF35 (**b**), anti-macrophage antibody HAM56 (**c**), anti-T200 antibody (**d**). Note the presence of a distinct fibrous cap and predominance of smooth muscle cells despite the presence of significant numbers of macrophages and lymphocytes. (**a–d**, original magnification, ×100) **e–g**—Different morphologic variants of HHF35-positive smooth muscle cells present in fibrous plaque. **e**—From fibrous cap area showing pancake-shaped cells near the lumen (*top*) with more plump, darkly immunostained cells in deeper intima. (Original magnification, ×125) **f**—High-power magnification of pancake-shaped cells. (Original magnification, ×375) **g**—Higher-power magnification of more plump, darkly immunostained cells. (Original magnification, ×250)

within the fibrous cap region in Figure 4c and d. The underlying or directly adjacent medial smooth muscle cells in some cases displayed a distinct gradient of antibody reactivity with the CGA7 anti-actin antibody, but not with the HHF35 anti-actin antibody or the anti-vimentin antibody. This gradient extended from the deep media to the intimal surface of the vessel, where staining intensity was weakest (Figure 5a and b).

The uniformly Factor VIII-positive and UEA-positive endothelium was present over the fibrous plaque only in a minority of cases, and generally in a patchy fashion (data not shown).

Advanced Plaques

On H&E-stained sections, advanced plaques were those that had very complex morphologic features, suggesting that they may have been modified by thrombosis and organization, dissection, and/or massive intimal thickening. Foam cells were generally present, although in variable numbers. Intact specimens of larger lesions could be divided into fibrous cap, shoulder, and atheromatous core zones. These lesions were those most frequently encountered in the carotid endarterectomy specimens (Figures 6a and d).

The advanced plaques displayed the greatest variation in cell type composition and distribution. The smooth muscle population displayed much of the same regional and morphologic variation described in the fibrous plaques; in the fragmented carotid endarterectomy specimens there was often noted a distinct "layering" phenomenon characterized by bands of HHF35-positive and CGA7-positive smooth muscle cells (Figure 6b). Although containing a predominant population of HHF35- and CGA7-positive smooth muscle cells, these lesions contained a relatively large population of blood-borne cells, although, as in the fibrous plaques, this varied from region to region and from plaque to plaque; these variations, however, were even greater than those seen in the fibrous plaques. Examples of the cell composition are given in Figure 6. Significant numbers of anti-T200-positive, HAM56-negative lymphocytes were present in these lesions as well, but in aggregate there were always more HAM56-positive (monocytes/macropahges) cells than HAM56-negative round cells, the latter presumably corresponding to lymphocytes.

Some regions, such as the lateral or "shoulder" zone of the advanced plaque, consistently contained great numbers of HAM56-positive macrophages and anti-T200-positive lymphocytes, which at least focally outnumbered the CGA7- and HHF35-positive smooth muscle cells (Figure 6e–g). Occasionally, vascularization of this portion of the lesion was noted; small vessels lined by Factor VIII- or UEA-positive cells were clearly delineated (Figure 6h). In contrast, in the overlying fibrous cap, the HAM56-positive macrophages corresponded to a small fraction of the cells; but as might be expected, toward the base of the lesion, where atheromatous debris was found, the number and proportion of HAM56-positive cells increased (data not shown). With the use of double-labeling techniques, two different types of foam cells were identified in the fibrous cap region; those that were HAM56-positive and presumably of monocyte-macrophage origin and those that were HHF35-positive and presumably of smooth muscle cell origin (Figure 8b). Both labels were never seen in the same cell.

In the media subjacent to a significant number of the more advanced plaques were areas of smooth muscle cell depopulation, ie, foci of absence of GCA7- and HHF35-positive cells (Figure 7a). The cells in these foci were, however, positive with the anti-vimentin antibody (Figure 7b); and, in addition, these foci contained Factor VIII-positive and UEA-positive endothelial cells demonstrative of vascularization (Figure 7c). Accompanying these endothelial cells were clusters of anti-T200-positive and HAM56-positive macrophages and lymphocytes (Figure 7d and e). These regions of medial vascularization were generally restricted to the outer third of the media but occasionally extended to the inner portions of the media as well. With these latter antibodies, foci of vascularization were also noted in the deep portions of advanced intimal plaques and occasionally within the shoulder regions of advanced plaques (see above).

With the anti-Factor VIII and UEA antibody studies, it was noted that more advanced plaques obtained from the endarterectomy specimens were devoid of an endothelial surface (data not shown).

Discussion

In this study we have demonstrated the feasibility of applying monoclonal antibody technology to the study of the lesions of human atherosclerosis. We have utilized hybridoma antibodies generated in our laboratory to muscle cell actins (HHF35), smooth muscle cell actin (CGA7), vimentin (43βE8), and macrophages (HAM56), together with other commercially available monoclonal and polyclonal antibodies to study the cellular composition of human atherosclerotic plaques. Each cell type in the vessel wall is characterized by a unique antibody profile (Table 4). Although studies with cell-type–specific monoclonal antibodies have been recently performed by other investigators,[13–17] we have been able to extend these results in the following ways: 1) Simultaneous documentation of the distribution of multiple cell types, including smooth muscle cells,

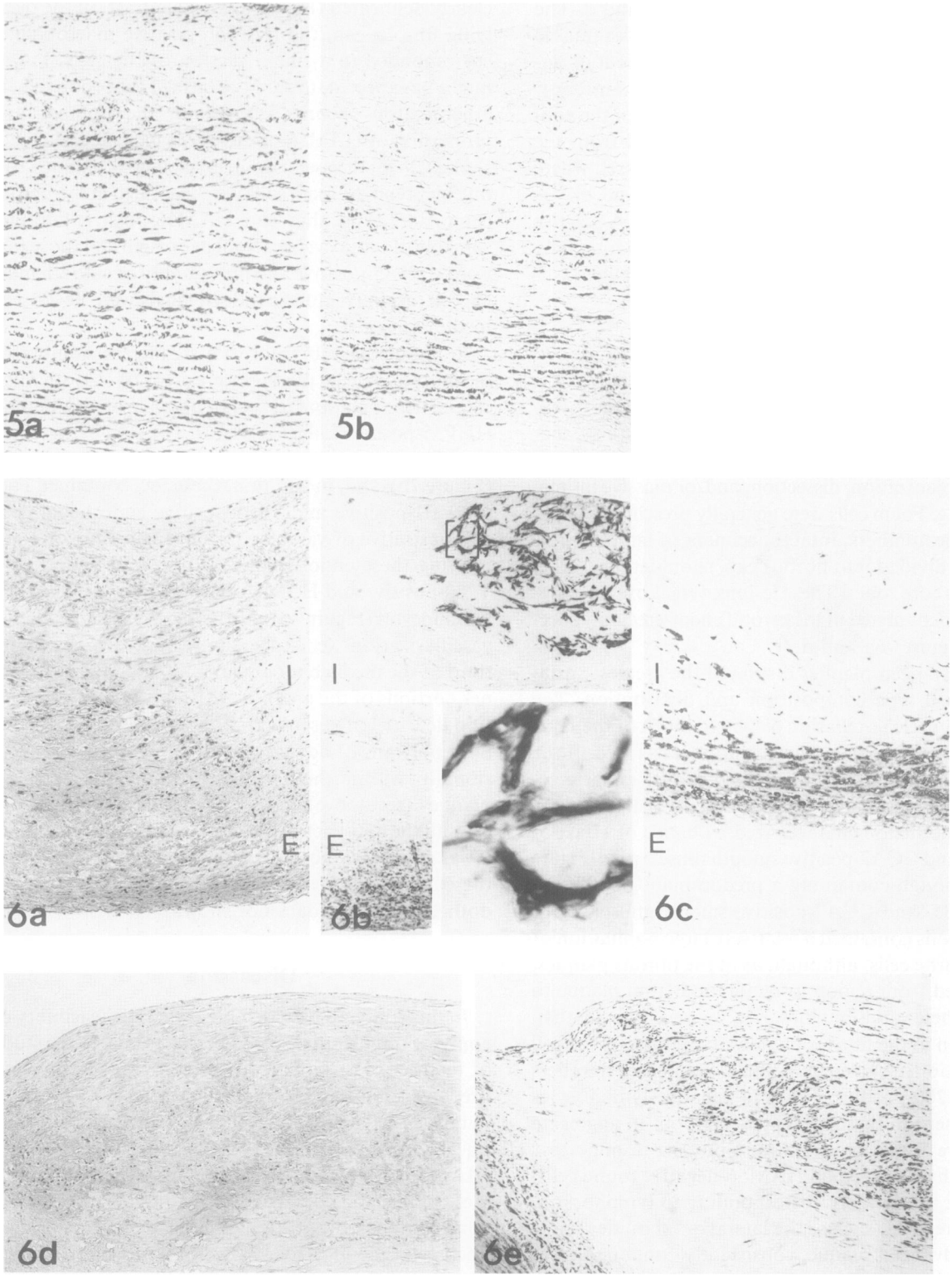
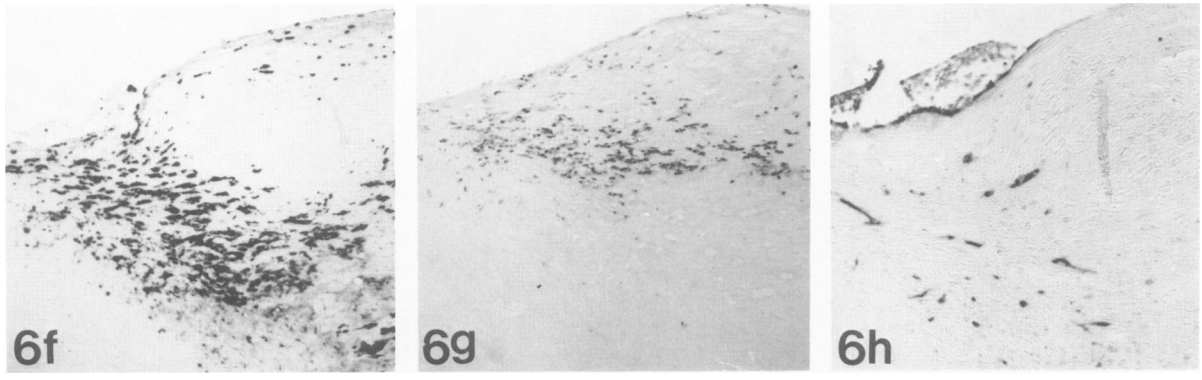


Figure 5—Aorta adjacent to a fibrous plaque demonstrating the gradient of smooth muscle cell immunoreactivity with antibody CGA7 (**b**) but not antibody HHF35 (**a**). The lumen is at the *top* of the figure. (Original magnification, ×60) **Figure 6**—Advanced plaque from carotid endarterectomy specimen (**a–c**) and shoulder region of aortic specimen (**d–h**). **a**—H&E-stained preparation. **b** and **c**—Serial sections reacted with anti-muscle cell antibody HHF35 and anti-macrophage antibody HAM56, respectively. Note "layering" of muscle cell and macrophage regions in plaque. (**a–c**, original magnification, ×50) **Inset** in **b**—Higher magnification of smooth muscle cell region reacted with antibody HHF35, note the dendritic morphologic character of the cells. (Original magnification, ×400) **d**—H&E-stained preparation. **e–h**—Serial sections reacted with anti-muscle cell antibody HHF35 (**e**), anti-macrophage antibody HAM56 (**f**), anti-T200 antibody (**g**), and UEA (**h**). Note large numbers of macrophages and lymphocytes present in this portion of



an advanced plaque and relative regional segregation of muscle cells and blood-borne cells. Note also the vascularization of this portion of the plaque. (**d–h**, original magnification, ×100)

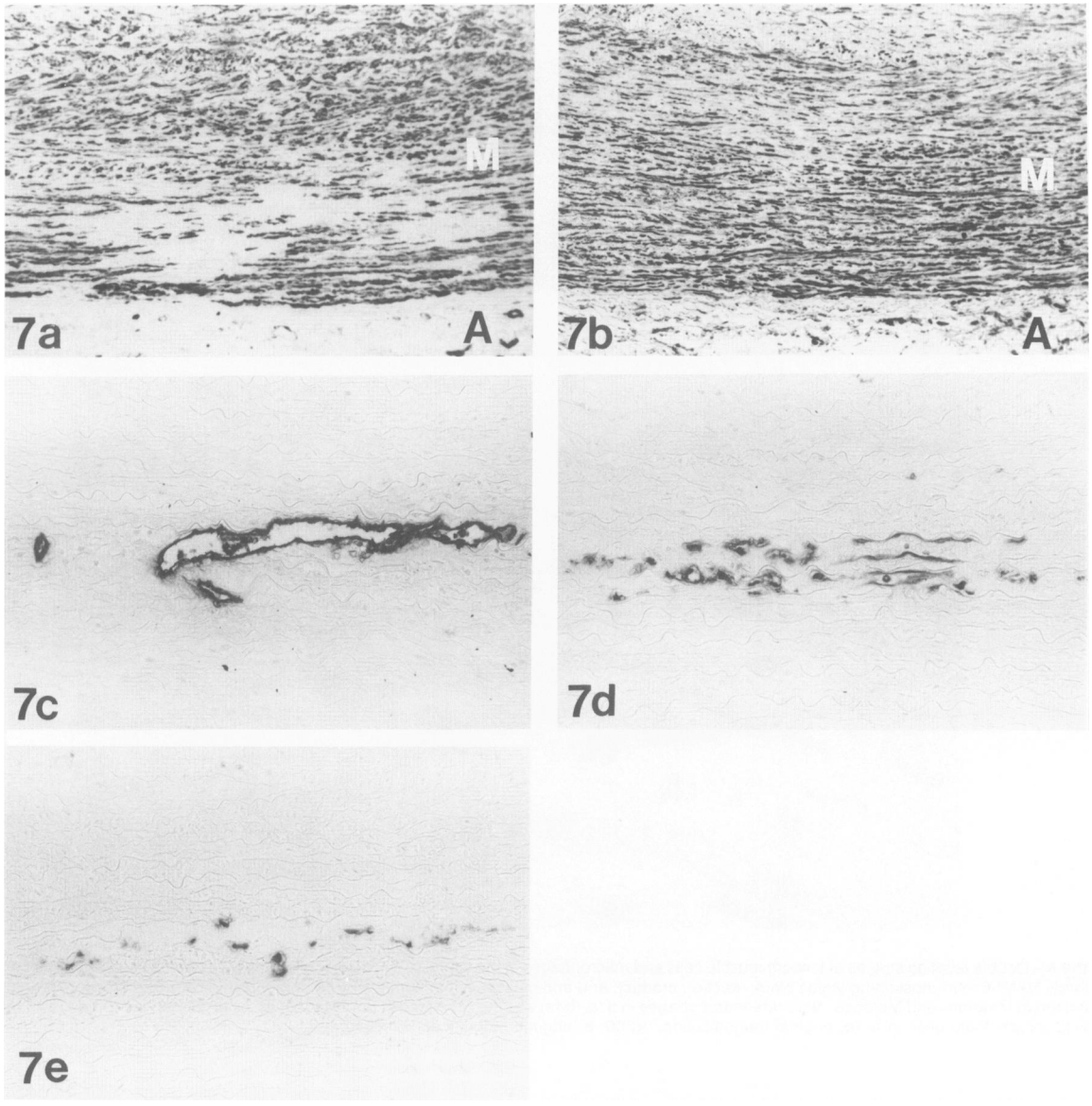


Figure 7—Aortic media subjacent to advanced plaque of abdominal aorta. Sections reacted with the following antibodies: anti-muscle cell antibody HHF35 (**a**), anti-vimentin antibody 43βE8 (**b**), UEA (**c**), anti-macrophage antibody HAM56 (**d**), anti-T200 antibody (**e**). Note striking vascularization of the media in **c**, which corresponds to the "holes" in the HHF35 immunoreactivity pattern of **a**. UEA-positive endothelial cells are characteristically accompanied by blood-borne cell infiltrates, as noted in **d** and **e**. Note vimentin-positive fibroblasts in the adventitia (**b**), which are nonreactive with antibody HHF35 (**a**). Medial "holes" are not apparent with anti-vimentin antibody, in contrast to anti-muscle cell antibody. (**a** and **b**, original magnification, ×80; **c–e**, original magnification, ×220)

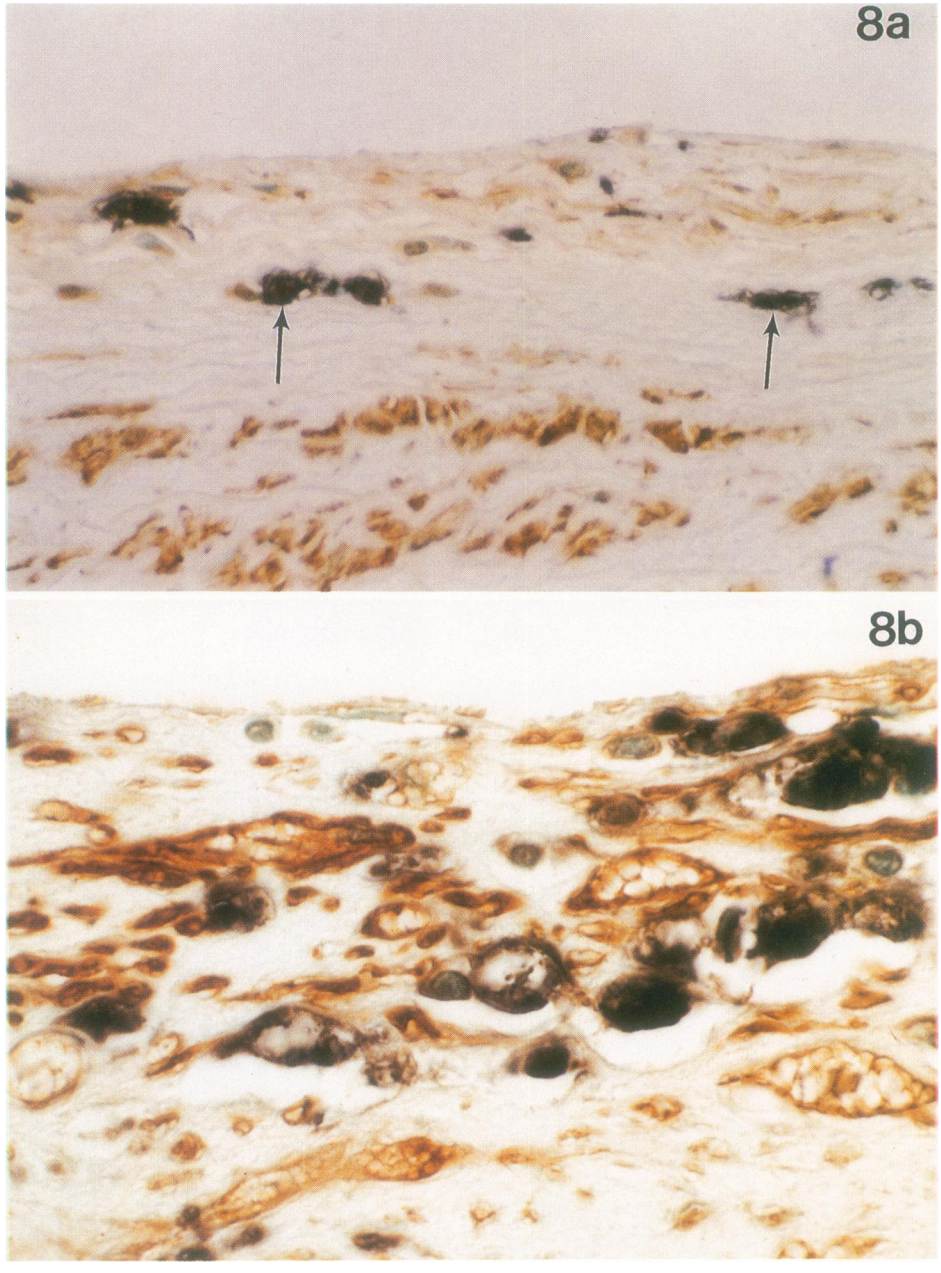


Figure 8—Double labeling studies of smooth muscle cells and macrophages in the intima of a nonlesional aorta (**a**) and fibrous plaque (**b**). Anti-macropahge antibody HAM56 immunostaining yields black reaction product, and anti-muscle cell antibody HHF35 immunostaining yields brown reaction product, as described in Materials and Methods. Note rare macrophages in **a** (*arrows*) and the presence of both smooth muscle cell-derived (*brown*) and macrophage-derived (*black*) foam cells in **b**. (**a**, original magnification, ×200; **b**, original magnification, ×240)

Table 4—Summary of Cell Identification by Monoclonal Antibodies

Cell type	43BE8	CGA7 HHF35	Anti-T200	HAM56	Anti-Factor VIII
Fibroblasts	+	-	-	-	-
Smooth muscle cells	+	+	-	-	-
Lymphocytes	-	-	+	-	-
Endothelial cells	+	-	-	+/-	+
Monocytes	-	-	+	+	-
Macrophages	+	-	+	+	-

monocyte/macrophages, lymphocytes, and endothelial cells within the lesions has been possible. 2) We have generated and employed monoclonal antibodies to the individual cell types which appear to react with the entire population of a given cell type. For example, our antibody HAM56 appears to react with all tissue macrophages and hence monocyte-derived foam cells; in the study by Watanabe et al[13] of rabbit lesions, their monoclonal antibody SRM1 did not appear to react with all foam cells within the lesions, but it is uncertain whether this is a function of a difference in cell origin (ie, are these smooth-muscle-derived foam cells) or failure of the monoclonal antibody to recognize what may be different functional classes of monocyte-derived macrophages. Preliminary studies in our laboratory, for example, with an antibody RAM11, an apparent counterpart to HAM56, generated to rabbit macrophages, demonstrates that even the cells negative with SRM1 are, in fact, positive with RAM11. The same can be said for the use of smooth-muscle-cell-specific antibodies, such as antibody YPC1/3.12 of Aqel et al.[16] There is no evidence presented in their paper to document the reactivity of this antibody on all smooth muscle cells. As with another smooth muscle marker protein, desmin, variations in expression as a function of cell state may call into question the utility of a given cell-specific marker in immunocytochemical studies (see below). 3) We have systematically examined the distribution of the various cell types in a number of types of human lesions and compared them with the nonatherosclerotic vessel. 4) We have employed antibodies that permit simultaneous preservation of antigenicity and intact morphologic features. While the studies of Aqel et al[14,16] required the use of frozen sections, our studies have been performed on methanol-Carnoy's-fixed, paraffin-embedded sections. Preliminary studies have also suggested that many of the antibodies employed here, including HHF35, HAM56, and the anti-T200 monoclonal antibody, can also be applied to aldehyde-fixed, paraffin-embedded sections; in future studies we plan to exploit this to perform ultrastructural immunocytochemical studies, which are not possible on alcohol-fixed tissue.

The new monoclonal antibody HAM56 described here, although its cell specificity appears comparable to that of commercially available antibodies such as Leu-M3, OKM1, and OKM5, has the additional benefits of 1) reacting with a cytoplasmic cell component and 2) reacting with these cells in fixed, embedded tissues. Although it appears to cross-react with rare nonmonocyte/macrophage cells such as renal tubular epithelium, this same cross-reactivity has been described with other macrophage-specific monoclonal antibodies.[35] In the context of the vessel wall, however, such cross-reactivities are inconsequential; HAM56 does not react with smooth muscle cells or fibroblasts. Although it does react with some vascular endothelium (as do other macrophage-specific monoclonal antibodies[36]), the latter can be distinguished from the macrophage population by virtue of both morphologic features as well as differential reactivity with antibodies to Factor VIII-related antigen and UEA. Immunoblots have been negative, and thus it has been impossible, to date, to further characterize the macrophage-associated antigen recognized by antibody HAM56. Further studies, however, are under way for investigation of the nature of the antigen.

Relatively few immunocytochemical studies of human atherosclerotic material have been published. Most of the previous studies have been directed at localizing extracellular matrix proteins[37,38] lipids or lipoproteins,[39-41] and immune complexes.[42] Although it has been almost 20 years since the first analysis of the cell composition of atherosclerotic lesions with cell-type-specific antibodies,[43] the development of reliable cell-specific markers awaited the relatively recent development of hybridoma antibody technology.[44]

Prior to the development of cell-specific monoclonal antibodies, various methods had been suggested by several authors to distinguish smooth-muscle-cell-derived from monocyte/macrophage-derived foam cells in various animal atherosclerotic lesions, including immunolocalization of lysosomal enzymes, histochemical localization of acid lipase or other lysosomal enzymes, and the demonstration of Fc- and C3-receptors, all as markers of monocyte/macrophage cells.[3,6-12] Monoclonal antibody studies have theoretic advantages over these other methods, because of their demonstrated exquisite specificities, together with their applicability to embedded tissues and the analysis of cells in situ. With the reagents such as the antibodies described in this paper, it is also possible to perform retrospective analysis of lesions. Most importantly, the selection of antibodies, the immunoreactivity of which is preserved in methanol-Carnoy's-fixed, paraffin-embedded sec-

tions, represents a significant advance over previous studies.

We have selected two monoclonal antibodies to actin isotypes as smooth muscle cell markers. From studies with these antibodies, and comparisons with other published smooth muscle markers, additional information regarding smooth muscle phenotypic variation can be gleaned. For example, Gabbiani and his colleagues, as well as Jonasson et al, have analyzed some human lesions with desmin antibodies.[17,44] These latter antibodies showed a cellular localization similar, but not identical, to that obtained with our monoclonal antibodies CGA7 and HHF35, which are directed to muscle-specific actin isotypes.[20,21] Gabbiani et al indicated that diminished desmin localization was observed in the cells of the intimal plaque, though no systematic study with that antibody has yet been reported; our preliminary results with monoclonal antibodies to desmin have demonstrated very patchy positivity even in the normal aorta (data not shown), which would preclude the use of this antibody as a generic smooth muscle marker. These same investigators have looked at intermediate filament expression in rat arterial smooth muscle cells following balloon catheter injury, finding transient loss of desmin expression,[46] raising further questions about the use of desmin antibodies as a smooth muscle marker, while suggesting the existence of phenotypic variation of smooth muscle cells as a function of proliferation. Chamley-Campbell et al[47] reported that the cells of the monkey intimal plaque showed "diminished" smooth muscle myosin immunostaining, though, again, this was an isolated observation. The simultaneous use of the two anti-smooth muscle cell antibodies CGA7 and HHF35 puts these latter studies in perspective: preliminary data in our laboratory using rat smooth muscle cells indicate that antibody CGA7, directed to smooth muscle actin isotypes and an excellent smooth muscle cell marker, demonstrates markedly reduced reactivity on these cells, in a fashion analogous to that of anti-desmin antibodies, during smooth muscle cell proliferation. Indeed, in these preliminary studies a "gradient" of reactivity was observed across these rat lesions analagous to the gradient of CGA7 immunoreactivity noted in the media of human arteries in these studies (Figure 5). In contrast, antibody HHF35 appears to react with smooth muscle cells in a more uniform manner independent of cell state. Antibody HHF35 may thus represent a marker of smooth muscle differentiation superior to either antibody CGA7 or antibodies to desmin. Nonetheless, further studies are required to fully elucidate the nature of these antibody-directed epitopes and their alteration in various cell states.

There is other morphologic evidence, presented here and elsewhere, that vascular smooth muscles constitute a heterogeneous group of cells. Several morphologic variants of smooth muscle cells were identified by Orekhov et al[48]; it may well be that some of the morphologic variants of HHF35-positive smooth muscle cells (eg, dendritic cells) reported in this *in situ* study correspond to one of the morphologic variants that Orekhov et al have observed in digested, explanted tissue. Further correlative studies will be required to see if this is a true correspondence. An additional intriguing finding in this study was the restricted loci of many of the morphologic smooth muscle cell variants. The dendritic smooth muscle cells, for example, were generally found near the surface of fibrous caps in advanced lesions; the plumper, more densely immunostained HHF35-positive smooth muscle cells were invaraibly found deep to the pancake-shaped shaped cells of the fibrous cap. The presence of these morphologic variants of smooth muscle cells raises additional questions: do these morphologic variants correspond to functionally altered cells, and do these variants have identical cell lineages? Although it is assumed that all smooth muscle cells within plaques have origins in medial smooth muscle cells,[1] the presence of such phenotypic variation, together with recent evidence that these muscle-specific markers are present in a population of bone-marrow–derived stromal cells,[49] might suggest that the current dogma is an oversimplification of a far more complex process.

One of the major findings of this study was the striking cell compositional heterogeneity of what were often grossly similar lesions. Microscopic heterogeneity of grossly similar atherosclerotic lesions is a phenomenon well described by Haust.[50] The lesions obtained for our study correspond to what Haust characterizes as "pearly white fibrous plaques" and "atheromatous plaques," which are definitions based upon gross characteristics. Although our fibro-fatty lesions and fibrous plaques, which are distinguished largely by their cell compositional differences, both would fit under the Haust's rubric of "pearly white fibrous plaques," we do not ascribe any temporal significance to these differences in cell composition, although one might speculate that the former lesions, by virtue of their predominant macrophage content, predate the largely smooth muscle fibrous plaque lesions. Extrapolating from morphologic studies, one might expect that lesions such as fatty dots and perhaps gelatinous lesions are also composed predominantly of macrophages[50,51]; nonetheless, the relationship of these lesions to our fibro-fatty lesions is uncertain. Further work will clearly be needed to apply the reagents and techniques of this study to these other precursor lesions.

An additional finding that warrants comment is the

rather striking, albeit focal, vascularization noted both within the media of the aorta and within the plaques themselves. According to the classic studies of Wolinsky and Glagov,[52] the normal infrarenal human aorta does not contain penetrating vasa vasorum, but our findings in atherosclerotic vessels corroborate the earlier findings of histologic and microangiographic studies by Geiringer,[53] Schutte,[54,55] and others and the very recent silicone injection studies of coronary arteries by Barger et al.[55] Although we did not find this medial vascularization in areas of grossly normal vessel, we did find it both subjacent and directly adjacent to areas of plaque involvement. The pattern of vascularization of the shoulder lesion could be argued as evidence of organizing thrombosis, but we also observed Factor VIII- and UEA-positive vessels within the deep layers of the intimal plaque, subjacent to areas that histologically were composed of fibrous plaque containing pancake-shaped smooth muscle cells (data not shown). The role played by this apparent neovascularization of the media and intima in plaque pathogenesis and development is uncertain, but it is an intriguing finding given the potential roles of macrophages and platelets in inducing this change.[56]

Other questions are raised, but not completely answered by these studies. A particularly thorny problem is that of the quantification of the various cell types present in the different lesions. Owing largely to the great variation from lesion to lesion and from region to region within plaques, quantification schemes are difficult to employ. Nonetheless, because this is a persistent question, it should be pursued by using three-dimensional reconstructive methods coupled with immunocytochemistry performed on serial sections; counting could be performed with a slide-based computer-assisted quantitative microscopy system.

Another important question pertaining to these studies is whether there is a subpopulation of cells not identified with any of the antibodies used in this study. The method of analysis of the study does not permit direct answers to this question, which would require either multiple-labeling procedures on intact sections or studies such as cytofluorometric analysis on fragments of arterial wall and plaque which are beyond the scope of this investigation and which suffer from their own intrinsic artifacts. In the double-labeling studies reported here, however, in which markers for muscle cell and marocphage differentiation were used together, to the limit of resolution of the methyl green nuclear counterstain method, no unstained nonlymphoid cells were noted; certainly we have been able to characterize all the foam cells present in the lesions. Regions devoid of staining with any of the antibodies did not contain identifiable nuclei; it is a reasonable conclusion that such areas were totally devoid of viable cells. It is possible, but unlikely, that postmortem autolysis could account for loss of immunostaining. Foci of complete nonreactivity were also found in the surgically obtained carotid endarterectomy specimens, which had been fixed immediately upon removal. Furthermore, no loss of immunostaining, using the monoclonal antibody panel of Table 1, has been seen in positive control autopsy-derived tissues within the 12-hour postmortem interval used in this study. Nonetheless, future studies should employ the use of corroborative ultrastructural and cell digestion studies in the analysis of these lesions.

Finally, it should be mentioned that many of the antibodies described herein, such as HHF35, CGA7, and HAM56, cross-react with corresponding cells in other species such as monkey and rabbit. Thus, with the use of these antibodies, comparative studies can be undertaken for a direct comparison of the cellular composition of experimentally derived lesions with that of the human plaque.

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