The Role of the Monocyte in Atherogenesis

I. Transition of Blood-Borne Monocytes Into Foam Cells in Fatty Lesions

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In a previous publication[1] the author and his co-workers demonstrated that atherosclerotic lesion development in the aorta of hypercholesterolemic pigs was preceded by intimal penetration of blood-borne mononuclear cells, and that medial smooth muscle cells were not involved in the formation of early fatty lesions in this model. The current study shows that aortic arch lesions do not progress beyond the fatty cell lesion stage for up to 30 weeks of a moderate cholesterol/lard diet, although they become more extensive in area. Mononuclear cells were found adherent to the endothelium, in endothelial junctions, and in the intima during this period, and were ultrastructurally identified as monocytes by the presence of peroxidase-positive granules (peroxisomes) in their cytoplasm. In addition, lesion areas with nonspecific esterase activity correlated well with Sudan IV staining. Intimal monocytes and altered intimal monocytes with an enlarged cytoplasm and containing a few lipid droplets were both shown to be phagocytic by their uptake of ferritin, which had penetrated the intima after intravenous injection. Circulating monocytes and those adherent to the endothelial surface did not contain ferritin in these animals. The results indicate that blood mononuclear cells associated with lesion formation in this model are, in fact, monocytes, which subsequently undergo transformation into macrophage foam cells in fatty streak lesions. The absence of medial cell involvement indicates that monocytes are the major foam cell precursor in these lesions. (Am J Pathol 1981, 103:181–190)

WE RECENTLY[1] described the morphologic characteristics of the intima at prelesion stages in swine fed an atherogenic diet. The study demonstrated that the earliest aortic lesions to develop were of a fatty streak type and appeared after 12–15 weeks of an atherogenic diet. Moreover, these earliest lesions were invariably associated with areas of spontaneously enhanced permeability, as demonstrated by the *in vivo* uptake of intravenously injected Evans blue dye.[1] The results demonstrated a preferential adherence and intimal penetration of blood-borne monocytes in lesion-prone areas at prelesion stages and suggested a more prominent role of the monocyte in foam cell formation than has previously been accepted. The present study was undertaken to examine the subsequent development of atherosclerotic lesions in the Evans blue model in swine, with particular emphasis on further defining the role of the monocyte in lesion formation.

Materials and Methods

Thirty-three Yorkshire pigs, 6 weeks of age (15–20 kg body weight) were fed a Purina Pig Chow diet containing 1.5% USP cholesterol and 19.5% lard (C/L).[1] Fifteen age-matched pigs were fed the Chow diet alone. The animals were killed 6, 12, 15, and 30 weeks after initiation of the diet, and tissue samples were examined by light and electron microscopy. All the animals were injected with Evans blue (0.1 mg/kg in isotonic saline) 3 hours before killing, exsanguinated,

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and perfusion-fixed with glutaraldehyde as previously described.[1,2] Samples of the aortic arch, thoracic, and abdominal aorta from areas of Evans blue uptake (blue areas) and no dye uptake (white areas) were excised from standardized sites for electron-microscopic (EM) studies. Both lesion and nonlesion areas were sampled. All EM samples were postfixed in osmium tetroxide and dehydrated in ethanol. Samples for transmission electron microscopy (TEM) were embedded in Spurr's resin, sectioned, and stained with 5% methanolic uranyl acetate and lead citrate[3] prior to being viewed in a Zeiss EM 10 electron microscope. One-micron plastic-embedded sections were cut and stained with methylene blue–azure II–basic fuchsin[4] for light-microscopic examination. Scanning electron microscopy (SEM) samples were critical-point-dried from CO_2, sputter-coated with gold, and viewed in an Etec Autoscan.

For histochemical studies at the light-microscopic level, buffy coats were examined, as well as blood mononuclear cells and granulated cells which had been separated from swine whole blood by centrifugation on Ficoll-Hypaque as described by Albrecht et al.[5] Smears were stained for nonspecific esterase and peroxidase by the use of the combined technique of Yam et al.[6] Canine and human blood samples were similarly treated. In addition, serial frozen sections of aorta from lesion and adjacent nonlesion areas were stained either with oil red O or for nonspecific esterase and peroxidase by the use of the techniques of Yam et al[6] and Kaplan,[7] respectively. Demonstration of peroxidase activity in blood monocytes and arterial sections at the ultrastructural level was accomplished with the use of the Graham–Karnovsky[8] technique, modified as described by Blinzinger et al.[9] Sections unstained or stained with uranyl and lead salts were examined.

Results

Fatty Lesions

As previously described, the earliest lesions detected in this model were small (3–6 mm in diameter), slightly raised focal lesions, which were seen after 12 weeks of the cholesterol/lard (C/L) diet. Such lesions were always found within the confines of Evans blue dye uptake (blue areas) in the arch, at intercostal orifices, or at and around the abdominal trifurcation. After 15 weeks on the atherogenic diet, raised fatty streaks positive for oil red O were present in the abdominal aorta, while blue areas in the arch showed diffuse raised fatty plaques (Figure 1). Up to 15 weeks, these lesions were always of a foam cell nature, and confined to the intima, with no evidence of medial cell involvement in the intima or engorgement of smooth muscle cells with lipid (Figure 2).

Three distinct cell types were present in early focal and later fatty streak lesions. Large (30–60 μ diameter) foam cells predominated, characterized by a lipid-engorged cytoplasm (Figure 3). Lipid droplets were large, nonmyelinated, membrane-bound inclusions which showed variable lipid saturation, as judged by their osmiophilia (Figure 3). Foam cells had no basement membrane and contained no organized cytoplasmic filaments. The Golgi apparatus was extremely well developed. Foam cells in fatty streaks up to 20 weeks appeared as healthy, metabolically active cells, as judged by their ultrastructure. Little cellular degeneration was observed in lesions before 30 weeks.

The second lesion cell type observed was identical to those previously described by us in the intima of blue areas in normal and prelesion swine, and in 12-week lesions.[1] These elongated cells, with numerous cytoplasmic extensions, were scattered throughout the thickened intima of the lesion. Their cytoplasm was largely undifferentiated, although a few contained myofilament tracts and occasional dense bodies, suggesting a smooth muscle cell origin. They did not accumulate extensive lipid, and frequently appeared degenerated (Figure 3).

The third type of cell present in fatty streak lesions were seen adherent to the luminal surface of the overlying endothelium (Figure 4), passing between endothelial cells (Figure 5) and in the intima, usually just below the endothelium (Figures 4 and 6), but occasionally deeper. A similar distribution of these cells was also observed at prelesion stages, predominantly in blue areas. Adherent cells frequently displayed cytoplasmic extensions that indented the plasma membrane of the underlying endothelial cells (Figures 4 and 7). All such cells showed the ultrastructural characteristics of monocytes, being typically 5–10 μ in diameter, with indented nuclei containing coarse peripheral chromatin. Their sparse cytoplasm contained numerous free ribosomes, a few large mitochondria, small vesicular elements, and a variable number of dense granules (Figures 4–6). Other intimal cells were observed that were similar but showed an enlarged, or hypertrophied, cytoplasm (Figure 8). These hypertrophied monocytes frequently contained one or two lipid droplets adjacent to a Golgi apparatus (Figure 9). None of these mononuclear cell types had a basement membrane or contained organized cytoplasmic filaments.

Fibrous Lesions

After 30 weeks on the atherogenic diet, raised fibroatherosclerotic lesions were found in the abdominal

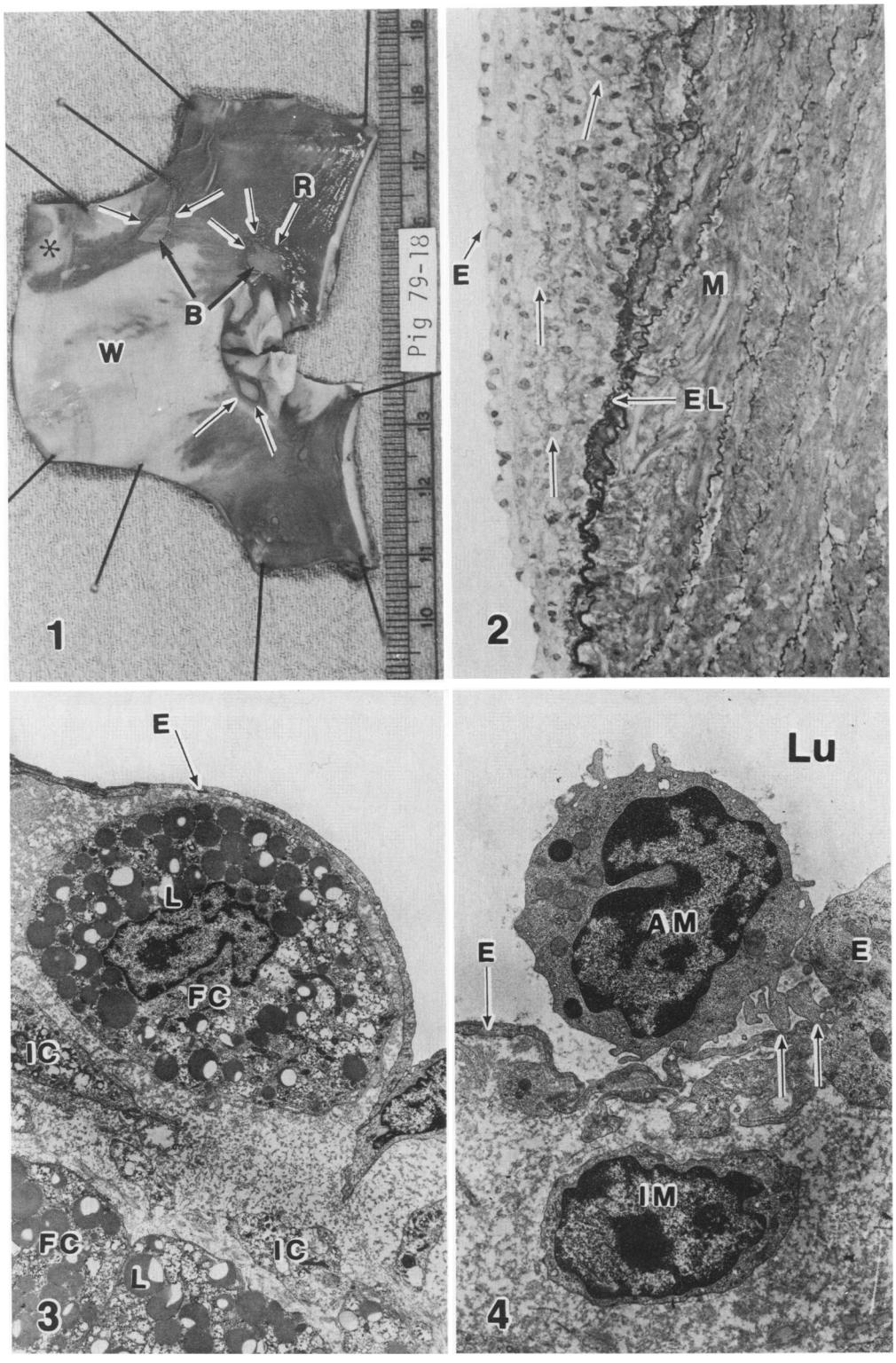

Figure 1—Aortic arch of a pig fed C/L diet for 15 weeks, stained with Sudan IV, showing extensive fatty streaking in areas which normally take up Evans blue, and extending down to closed ductus scar (*). The greatest lipid staining occurs at the periphery (*arrows*) of areas of heaviest blue uptake (*B*). These areas are raised from surface. White areas (*W*) show little or no Sudan staining. Lesion at *R* shown in Figure 2. **Figure 2**—Light micrograph of one epoxy section of raised lesion indicated by *R* in Figure 1. Intima is thickened with numerous lipid-laden cells (*arrows*) luminal to internal elastic lamina (*EL*). Media (*M*) shows no lipid accumulation, and endothelium (*E*) is intact. (Methylene blue-azure II-basic fuchsin, ×150) **Figure 3**—TEM of lesion similar to above showing attenuated endothelium (*E*) overlying foam cells (*FC*) in intima containing large lipid droplets (*L*). Partially degenerate intimal cells containing little lipid are also visible (*IC*). (Uranyl acetate, lead citrate, ×2800) **Figure 4**—TEM of 30-week arch lesion showing a monocyte (*AM*) in the lumen (*Lu*) adherent to endothelium (*E*) and an intimal monocyte (*IM*) subjacent to endothelium. Extensions (*arrows*) from adherent monocyte indent endothelium. (Uranyl acetate, lead citrate, ×7400)

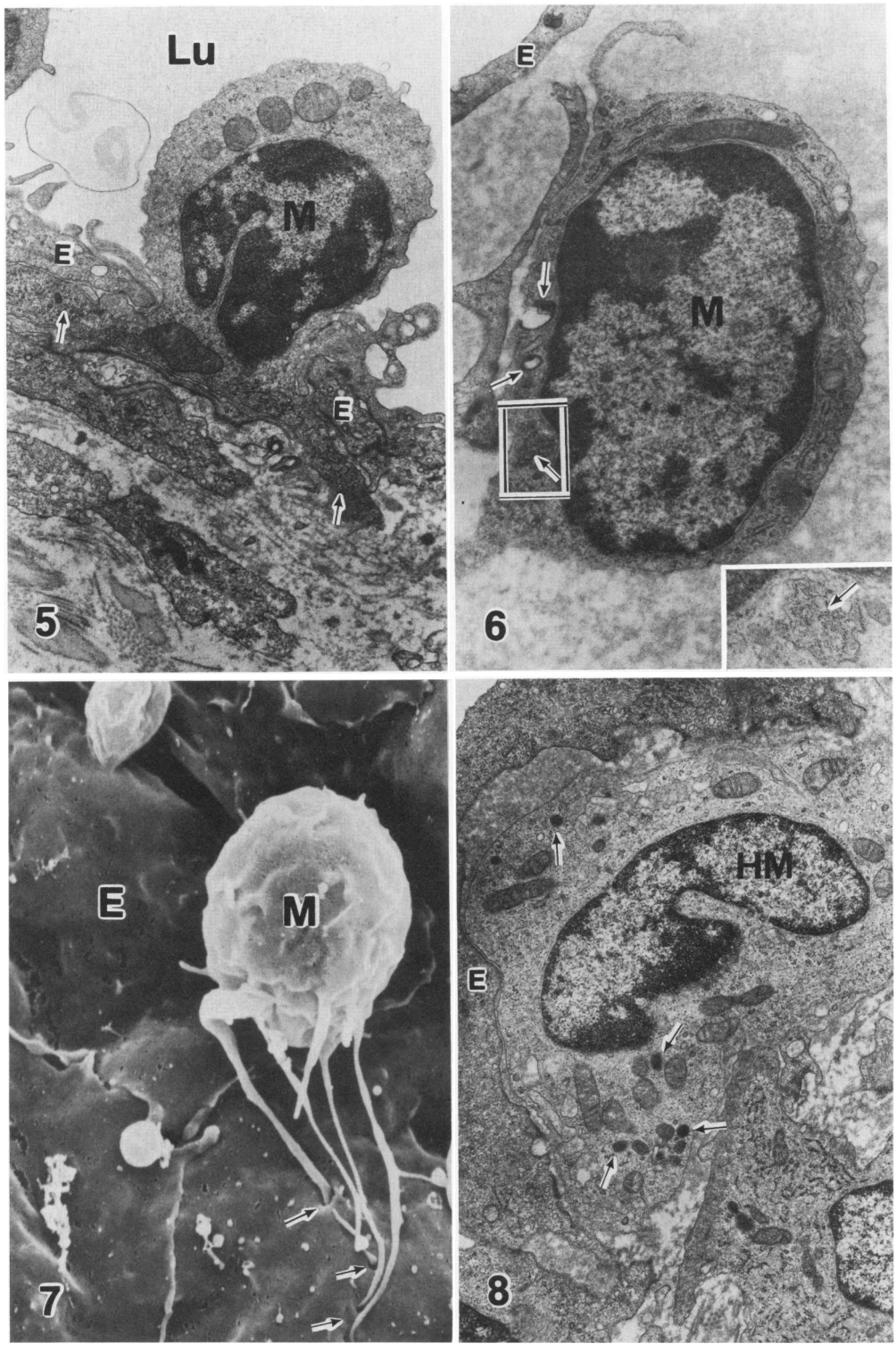

Figure 5—TEM of monocyte (*M*) trapped in junction of endothelium (*E*) from arch blue area of 12-week C/L-fed pig. Main body and nucleus of cell are in the lumen (*Lu*), with celular extensions (*arrows*) spread below endothelium. (Uranyl acetate, lead citrate, × 12,400) **Figure 6**—Monocyte (*M*) beneath endothelium (*E*) in a 15-week arch lesion from a pig injected with ferritin 15 minutes before death. Ferritin can be seen in phagocytotic vacuoles (*arrows*), one of which is open to the intimal space. **Inset** shows ferritin in vacuole in squared area. (Unstained, × 15,500; *Inset*, × 61,000) **Figure 7**—SEM of monocyte (*M*) adherent to endothelium (*E*). Cytoplasmic extensions from monocyte can be seen to indent endothelial plasma membrane (*arrows*). Compare with Figure 4. (× 7750) **Figure 8**—TEM of "hypertrophied" monocyte (*HM*) beneath endothelium (*E*) in 30-week C/L abdominal lesion. Peroxidase-positive granules (*arrows*) can be seen in the cytoplasm. (Uranyl acetate, lead citrate; peroxidase-reacted, × 12,500)

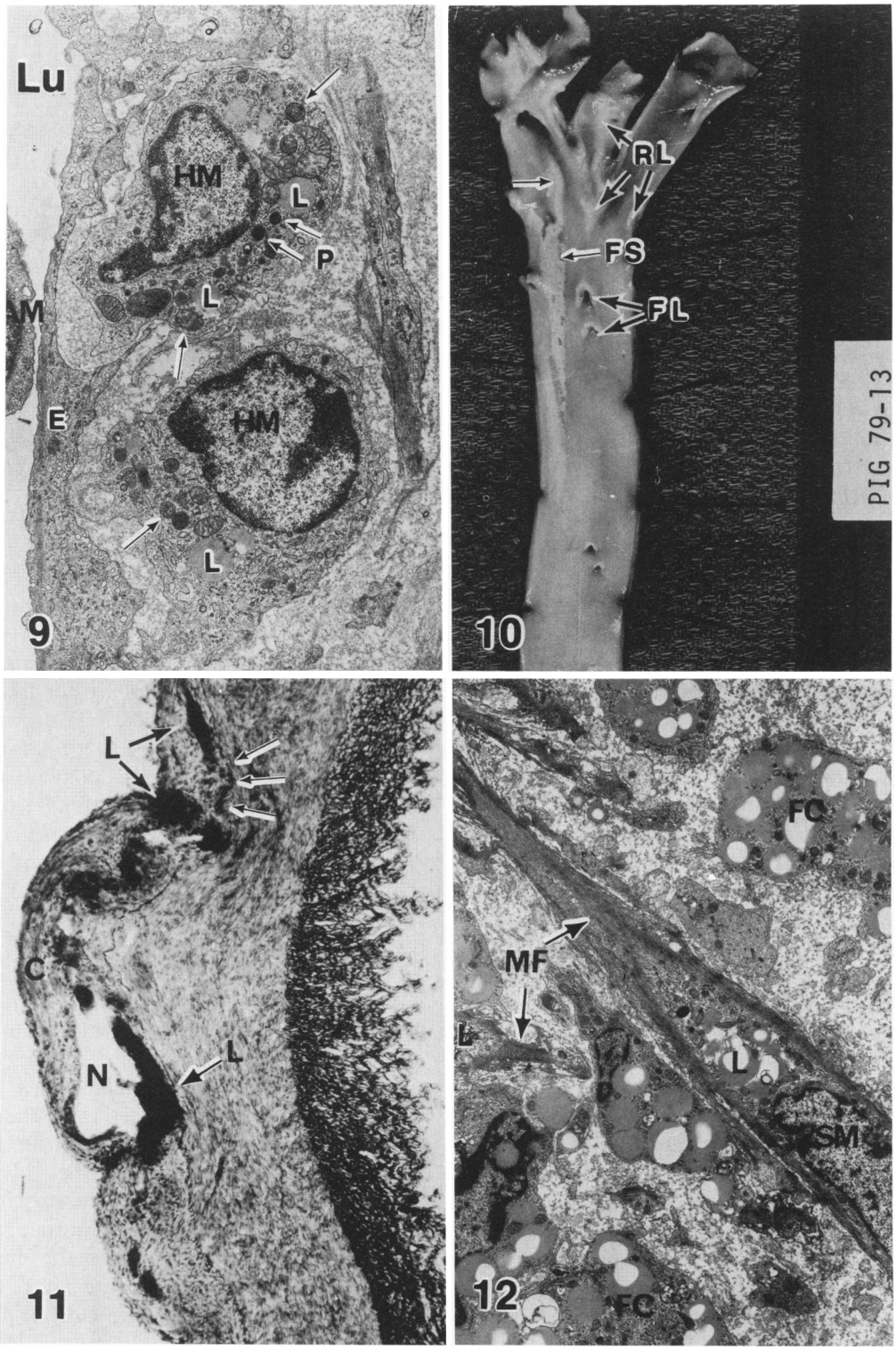

Figure 9—TEM of arch lesion, 30-week C/L-fed, ferritin-injected pig. Two intimal hypertrophied monocytes (*HM*) contain a few lipid droplets (*L*), and ferritin in vacuoles (*arrows*). Faint peroxidase activity (*p*) is present in one cell. An adherent monocyte (*AM*) is partially visible in the lumen (*Lu*) adjacent to the endothelium (*E*). (Uranyl acetate, lead citrate, peroxidase-reacted, ×8000) **Figure 10**—Abdominal aorta of a pig fed C/L diet for 30 weeks. Typical fatty streak (*FS*) terminates in early raised fibrous lesion (*arrow*) extending into right iliac artery. Other raised lesions originating at trifurcation are also visible (*RL*), and raised fatty lesions are present at other branch sites (*FL*). **Figure 11**—Light-photomicrograph of lesion marked with *arrow* in Figure 10. Necrotic core (*N*) is covered by a thin fibrous cap (*C*) and contains stainable lipid (*L*). Disruption of the internal elastic lamina is visible at *arrows*. (Oil red O, ×37) **Figure 12**—TEM from core of lesion similar to Figure 11, showing smooth muscle cells (*SM*) containing lipid droplets (*L*) and organized myofilaments (*MF*), as well as foam cells (*FC*) not recognizable as smooth-muscle-derived. (Uranyl acetate, lead citrate, ×4000)

aorta. These lesions occupied the same geographic location in the aorta as did earlier fatty streaks but often extended into the iliacs (Figure 10) and contained less oil-red-O–positive material. Histologically, these plaques were more fibrous, frequently with a fibrous cap overlying a necrotic lipid core (Figure 11). Monocytes were generally seen only in the immediate subendothelial layer, and lipid-laden smooth muscle cells (Figure 12), and necrotic foam cells (Figure 13) were the predominant lesion cell types.

Histochemistry

In an effort to further characterize these lesions and the cells within them, histochemistry at the light- and electron-microscopic levels was carried out. Blood mononuclear cells were separated from whole blood, and smears were stained for nonspecific esterase and peroxidase activity. Two cell types were distinguishable: one that was light yellow in color (esterase- and peroxidase-negative) and a second that was strongly esterase-positive (brick red), with variable peroxidase (blue-green) activity (Figure 14). Matching smears stained with Wright's stain showed over 99% mononuclear cells.

When adjacent serial frozen sections of fatty streak (15-week) lesions were stained with either oil red O or for esterase/peroxidase activity, oil-red-O–positive areas were also found to be strongly esterase-positive (compare Figures 15 and 16). The underlying media was both oil-red-O– and esterase/peroxidase-negative (Figures 15 and 16). Peroxidase activity was at best weak in these sections and was visible only under oil immersion, largely masked by the strong esterase reaction. Lesion sections stained for peroxidase alone likewise showed weak peroxidase activity, usually directly below the endothelium.

Although peroxidase activity was weak in frozen sections, it could be demonstrated ultrastructurally in monocytes separated from blood (Figure 17), adherent monocytes (Figure 18), and intimal monocytes (Figure 19). Moreover, it was present in most, but not all, of the "hypertrophied" intimal monocytes, both lipid-containing (Figure 9) or not (Figure 8). In all cases, peroxidase activity was confined to small, membrane-bound inclusions, or peroxisomes. The number of peroxisomes in swine monocytes separated from whole blood was usually 1–3 per cell in section, fewer than in human or canine blood controls separated and stained at the same time. Likewise, swine monocytes showed lesser peroxidase activity at the light-microscopic level.

In addition to containing one or two visible lipid droplets and peroxisomes in section, lipid-containing "hypertrophied" intimal monocytes in 15-week cholesterol-fed swine that had been intravenously injected with ferritin also contained vacuoles filled with phagocytosed ferritin (Figure 20). Ferritin was visible in these cells and in non-lipid-containing monocytes (Figure 6) within one minute after injection, and the number of ingested particles continued to increase up to 15 minutes after injection. It was not observed in adherent monocytes.

Advanced 30-week plaques showed considerably less nonspecific esterase activity than earlier fatty lesions, generally confined to the subendothelial layer. Peroxidase activity was again weak, although peroxisomes could be found in intimal monocytes at the ultrastructural level.

Discussion

We have previously demonstrated[1] that cells interpreted as being monocytes by their ultrastructural appearance can be seen adherent to the aortic endothelium and in the intima at early prelesion stages in cholesterol/lard-fed swine. Moreover, we quantitatively showed that these events occur preferentially in areas of Evans blue dye uptake and are not associated with endothelial damage. We undertook the present study to identify positively the invading cell type, since we did not feel that ultrastructure alone was a sufficient identification criterion, and, secondly, to determine the fate of intimal monocytes. The results of the present study demonstrate that adherence and intimal penetration by these cells continues to occur throughout the course of lesion development, although it is particularly prevalent in fatty streaks. Our belief of intimal penetration is based on the indirect evidence that monocytes are not prevalent in the intima of normal animals, they are not seen in the media, and their presence in the intima is concurrent with the adherence of monocytes to the endothelium and their being found trapped in endothelial junctions. The swine has proven to be an excellent model for this study, since lesions in the arch seldom progress beyond the fatty cell stage, although they become more extensive in area, whereas those in the abdominal aorta progress to fibroatheromatous plaques under the dietary conditions applied. Comparison of the plaque types within each animal is therefore possible; but more importantly for the present study, fatty cell lesions can be studied in a model in which they do not progress.

The histochemical demonstration of nonspecific esterase and peroxidase activities in mononuclear cells separated from whole blood in the present study is similar to that demonstrated by numerous investigators.[5,6,9–14] In particular, Yam et al[6] and Albrecht et al[5] have shown that of the two mononuclear cell types

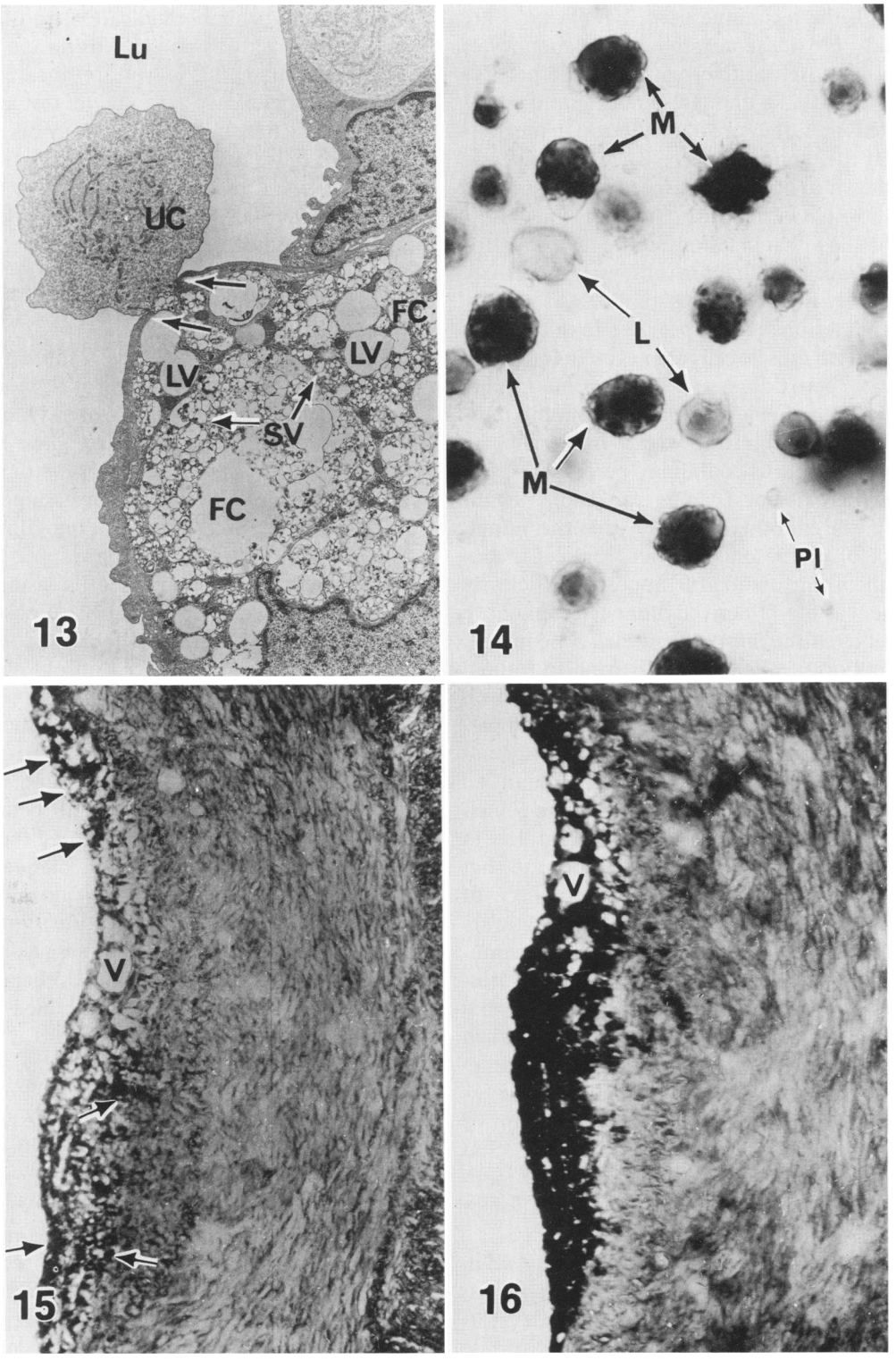

Figure 13—TEM of abdominal lesion, 30-week C/L diet-fed pig. Foam cells (*FC*) are much more necrotic in appearance than at earlier stages, containing many small membranous vacuoles (*SV*) and large clear vacuoles (*LV*). Lipid droplets in these cells did not show variable osmiophilia. Also visible is an unidentifiable cell (*UC*) extending through an endothelial junction into the lumen (*Lu*) at *arrows*. (Uranyl acetate, lead citrate, ×4800) **Figure 14**—Light-photomicrograph of Ficoll separated mononuclear cells from blood of 15-week C/L-fed pig. Monocytes (*M*) show dense but variable esterase (dispersed) and peroxidase (granular) staining, while lymphocytes (*L*) and platelets (*Pl*) are esterase/peroxidase-negative. (Esterase/peroxidase-reacted, ×1000) **Figure 15**—Light-photomicrograph of frozen section from a 15-week lesion. Esterase activity is confined to the lesion area (*arrows*). Vacuolated cells (*V*) serve as reference point. Compare with Figure 16. (Esterase/peroxidase-reacted, ×90) **Figure 16**—Light-photomicrograph of frozen section adjacent to that in Figure 15 stained to show oil red O activity, which is superimposed on areas of esterase activity shown in Figure 15. Vacuolated cells (*V*) serve as reference point. (Oil red O, ×90)

separated with the technique used, lymphocytes are esterase- and peroxidase-negative, while monocytes are strongly esterase-positive, with variable positive peroxidase activity. Although some granulocytes show both activities, they are of no interest in the present study. Of the mononuclear cells, only monocytes have been shown to be both nonspecific esterase- and peroxidase-positive with the use of these techniques.[5,6]

The results show that in lesions in which all identifiable lipid is intracellular in foam cells, esterase activity correlates closely with oil red O staining. As already discussed, arch lesions do not progress in this model, the medial smooth muscle cells are negative for oil red O and esterase/peroxidase, and there are no intimal cells recognizable as being derived from smooth muscle in these lesions for up to 15 weeks. In the same lesions, mononuclear cells containing peroxidase-positive granules (peroxisomes) can be found adhering to, or traversing, the endothelium and within the intima. The presence of peroxisomes within these cells, coupled with their ultrastructural appearance, clearly identifies them as blood-derived monocytes that have apparently entered the intima from the aortic lumen. Of greater importance is that hypertrophied intimal mononuclear cells, which contain some lipid and which phagocytose ferritin, also can be shown to contain peroxisomes, indicating a monocytic origin. These findings clearly show that intimal monocytes (identified by their peroxisomes) are phagocytic at a time coincident with the initial appearance of lipid droplets in their cytoplasm. The results therefore indicate that blood-derived monocytes are the prime source of foam cells in these early lesions. Their phagocytic activity suggests that intracytoplasmic lipid is accumulated through phagocytosis. The other cell type present—the undifferentiated cell found even in normal animals[2]—is also phagocytic[10] but can be found in these lesions free of intracytoplasmic lipid. The penetration of the intima by monocytes is much more extensive in areas of enhanced intimal penetration of blood molecules,[1] as shown by both Evans blue[1,2] and ferritin uptake.[10,11] This invasion by phagocytic monocytes may be in response to a greater accumulation of intimal lipid in these areas.

The foam cells of these lesions have been shown in the present study to be strongly esterase-positive, but are peroxidase-negative at the ultrastructural level. It can be assumed, therefore, that the weak peroxidase reaction seen just below the endothelium in frozen sections is attributable to intimal monocytes. The peroxidase negativity of monocyte-derived foam cells is not surprising, since it is generally accepted that blood-borne monocytes lose their peroxidase activity after entering the tissues and becoming actively phagocytic.[12] The hypertrophied intimal monocytes of the present study probably indicate a transitional form, since they are actively phagocytosing and beginning to accumulate lipid, but as yet are not lipid-laden. As such, they apparently have not yet lost all peroxidase activity, which is the case when they become lipid-laden foam cells. It would appear from our studies as well that swine monocytes have fewer peroxisomes than either human or canine blood. This belief is further substantiated in the literature, where studies using similar techniques on other species have demonstrated more numerous peroxisomes in monocytes than we observed in swine.[7,9,13,14]

The presence of both smooth muscle and macrophage foam cells in atherosclerotic plaques has long been recognized,[15-19] but until recently, only the studies of O'Neal and co-workers[20-23] have attempted to establish macrophages as a major factor in lesion formation. Their source has been assumed to be the blood monocyte ever since Poole and Florey[15] demonstrated a macrophage in section trapped between two endothelial cells. Their presence in lesions has, however, largely been considered as almost incidental, since the medial smooth muscle cell undoubtedly plays the major role in the advanced fibrous plaque[24,25] and its importance has been emphasized repeatedly. In recent years, however, the circulating monocytes and tissue macrophages have been shown to be capable of actively synthesizing cholesterol[26] and metabolizing lipid.[27,28] The development of histochemical techniques has increasingly demonstrated the presence of macrophages in various types of plaques,[29-32] which has been related to a role in lipid removal.[29] Gaton and Wolman[31] demonstrated a stratification of two cell types in atheroma. Cells with intense acid esterase activity were aligned under the endothelium, whereas cells deeper in the lesion showed much less activity. They interpreted the esterase-rich cells as being blood-derived macrophages[30] and the enzyme-poor cells as being derived from smooth muscle. Both cell types were lipid-laden, but lipid in esterase-rich cells was finely emulsified, whereas acid-esterase-poor (smooth muscle) cells contained bulky aggregates of birefringent lipid, indicating a relative inability to metabolize lipid. The authors concluded that the relative insufficiency of acid esterase in myocytes may play an important role in atheroma development, and that macrophages could conceivably remove lipid, changing atheromata to fibrous plaques. Adams and Bayliss[30] confirmed the paucity of macrophage foam cells in rabbit atherosclerotic lesions, as is the case in humans,[33] and similarly suggested that lesions from which lipid is rapidly removed are rich in macrophages.

The present study demonstrates that in swine fatty streak lesions that do not progress, monocyte-derived

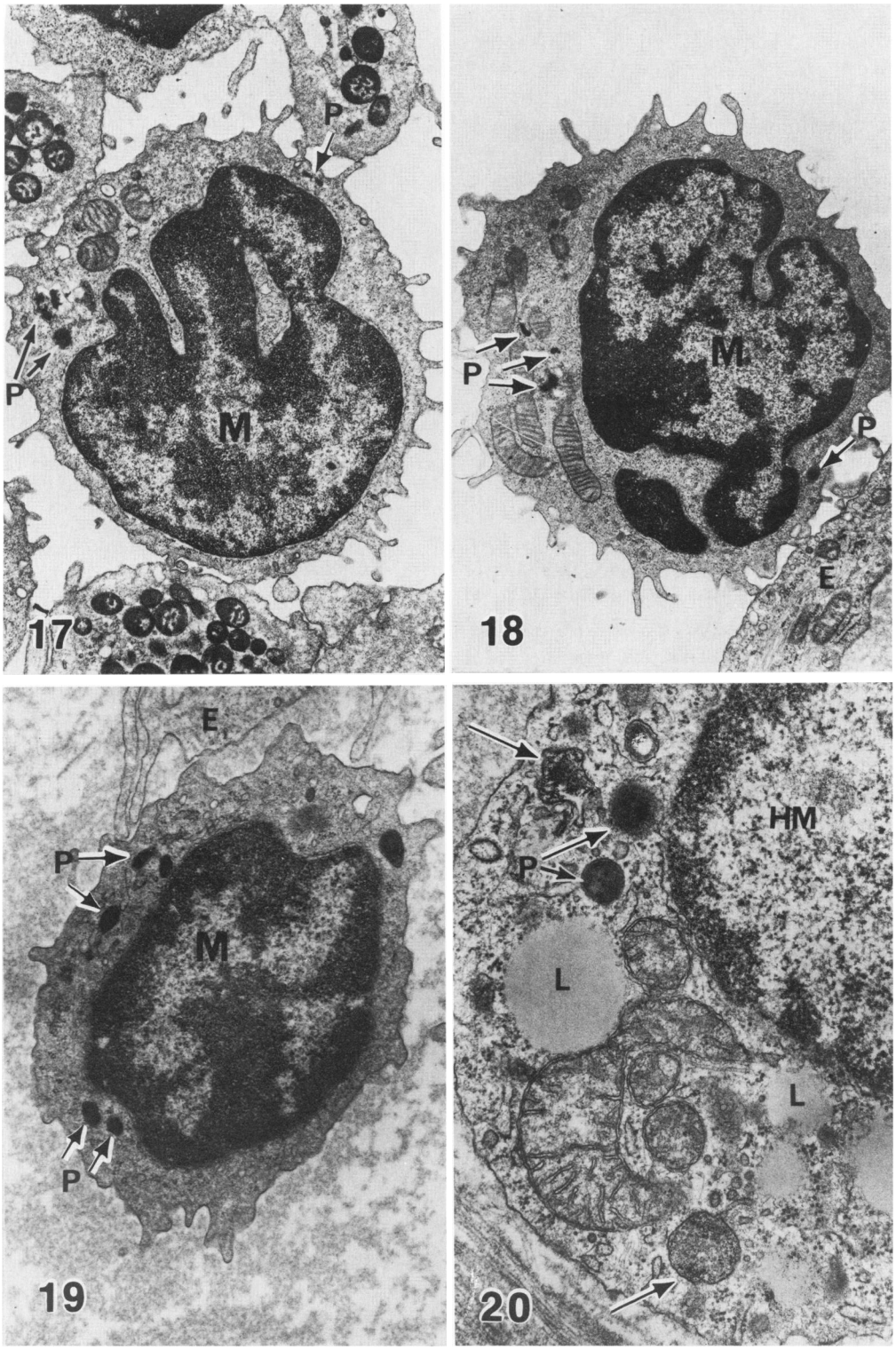

Figure 17—TEM of monocyte (*M*) in buffy coat from normal pig blood. Peroxisomes (*P*) are visible in cytoplasm. (Peroxidase-reacted; uranyl acetate, lead citrate, × 10,000) **Figure 18**—TEM of monocyte (*M*) adherent to aortic endothelium (*E*) in 6-week C/L-fed pig. Peroxisomes (*P*) are visible in cytoplasm. (Peroxidase-reacted, uranyl acetate, lead citrate, × 12,000) **Figure 19**—Monocyte (*M*) containing peroxisomes (*P*) in intima below endothelium (*E*). (Peroxidase-reacted; uranyl acetate, lead citrate, × 15,000) **Figure 20**—TEM of cytoplasm of "hypertrophied" intimal monocyte (*HM*) containing lipid droplets (*L*), peroxisomes (*P*), and phagocytic vacuoles containing ferritin (*arrows*). (Peroxidase-reacted; uranyl acetate, lead citrate, × 30,500)

macrophages are the major source of lesion foam cells. In advanced abdominal lesions, the smooth muscle foam cell predominates. In view of other studies,[27-31] we are tempted to suggest that the lack of progression of arch lesions is due to their high content of esterase-rich macrophage foam cells, which are capable of metabolizing lesion lipid. The fact that we have examined swine aortas from the onset of lipid feeding, together with the fact that arch lesions under the conditions used did not progress, allows us to demonstrate, ultrastructurally and histochemically, a major role of the monocyte in the formation of fatty cell lesions. The areas of enhanced permeability in which the earliest lesions form perhaps contribute to large numbers of migrating monocytes in response to enhanced lipid accumulation at these sites.

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