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Aortic intimal monocyte recruitment in the normo and hypercholesterolemic baboon (*Papio cynocephalus*)

An ultrastructural study: Implications in atherogenesis

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Summary. The ultrastructural features of peripheral blood monocyte margination, migration, and aortic intimal accumulation have been described in the normo- and mildly hypercholesterolemic baboon. Intimal monocyte-macrophage recruitment over fatty streaks and fibro-fatty plaques was enhanced by dietary cholesterol-fat supplementation, resulting in an 8-fold increase in monocyte-macrophages and macrophage-derived foam cells in the subendothelial space. Margination or attachment observed over both plaques and normal areas was not associated with morphologic evidence of endothelial injury. Migration through continuous aortic endothelium was principally between endothelial cells via junctions. Transitional sequences from the typical morphology of the blood monocyte to the lipid-containing macrophage or foam cell were discerned. The intimal accumulation of monocytes and macrophages reinforces our view of atherosclerosis as an inflammatory process, in which monocyte attachment is likely to reflect changes in the endothelial surface-membrane complex and surface charge, while migration to and accumulation in the SES may result from one or more chemoattractants originating in the intima or media.

Key words: Peripheral blood monocytes – Macrophages-Foam cells – Attachment – Migration – Atherogenesis – Inflammation

Introduction

Peripheral blood monocytes are the progenitors of tissue macrophages (Van Furth 1970) including the Kupffer cell of the liver, alveolar and peritoneal macrophages, and microglial cells. These macrophages play an important role in inflammation serving both as scavengers and secretory cells (Stossel

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1974; Unanue 1976), and also as regulators of lymphocyte function (Unanue 1978 and 1979). The relative contributions of monocyte-derived macrophages and smooth muscle cells, as precursors of the lipid-rich intimal or plaque foam cell population seen in atheroma, have long been the subject of debate and are still to be resolved. Duff et al. (1957) convincingly described monocytoid cells on and below the aortic endothelium in cholesterol-fed rabbits, a finding which has been confirmed in the cholesterol-fat-fed pig (Schwartz et al. 1978; Gerrity et al. 1979; Gerrity 1981), baboon (Schwartz et al. 1980a), rat (Joris et al. 1983) and pigeon (Lewis et al. 1982). Because of the potential roles of monocyte-derived macrophages as a source of arterial wall proteases such as elastase, as precursors of at least some arterial wall foam cells, and as the source of macrophage-derived growth factor (Leibovich and Ross 1976; Greenburg and Hunt 1978), their participation in atherogenesis could assume a new significance. This study describes selected quantitative light microscopic and ultrastructural features of blood-derived monocytes, macrophages, and macrophage foam cells in the aortic intima of the normal-fed and mildly hypercholesterolemic cholesterol-fat-fed nonhuman primate (*Papio cynocephalus*).

Materials and methods

Animals. Aortas from 16 adult baboons (*Papio cynocephalus*) of both sexes ranging in estimated ages between 5 and 20 years were studied. Of these, seven were mildly hypercholesterolemic (range 157–236 mg/dl), having received either a cholesterol-coconut oil-peanut oil and egg yolk-supplemented diet for 5 years (4 animals) or a cholesterol-lard-egg yolk diet for 2 years (3 animals). The remaining 9 were normocholesterolemic (range 42–185 mg/dl) having received chow with neither cholesterol nor fat supplements.

Perfusion fixation. All animals were perfusion-fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, at an osmolality of 300 mOsm. The buffered glutaraldehyde was infused at a pressure of 100 mm Hg for 30 min after first flushing the circulation with Krebs-Ringer solution (pH 7.3, 280 mOsm) at 37° C for 5 min maintaining a pressure of 100 mm Hg.

Electron microscopic procedures. Aortas were carefully removed from the animal to avoid distortion and opened using 2 lateral incisions, thus minimizing mechanical damage to the relatively rigid cylinders. Samples for both transmission and scanning electron microscopy were routinely excised with a razor blade from 5 standard sites in the arch, thoracic, and abdominal aortic segments, together with selected samples exhibiting either fatty streaks or fibrous plaques. Care was taken to avoid air drying of the tissues. Samples for scanning electron microscopy, measuring approximately 1–2 cm^2, were post-fixed in 1% buffered osmium tetroxide, dehydrated in ethanol, critical-point dried in a Bomar SP 1500 using Freon 13, and lightly coated with palladium-gold in a Hummer II instrument for viewing in a Philips 500A. The smaller samples for transmission electron microscopy were post-fixed in buffered 1% Osmium tetroxide and 1.5% potassium ferrocyanide, en bloc stained with 3% methanolic uranyl acetate, and embedded in araldite. One micron sections were stained with 1% Azure II-methylene blue. Ultrathin sections were further stained with lead citrate, prior to examination in a Philips 301 or a JEOL 100 CX electron microscope.

Results

Light microscopy. Table 1 presents quantitative cellular data derived from one micron sections obtained from a subset of 8 animals. Mononuclear

Table 1. Surface and intimal cellularity

Animal number	Diet category	Terminal plasma cholesterol (mg/dl)	Number of 1 μ sections examined	Mean intimal thickness (μ)	Surface and intimal cellularity		
					Attached surface[a] monocytes (cells/1,000 μ)	Cells* in the SES (cells/1,000 μ)	Total intimal cellularity**
2416	Low	107	13	9	1.80	0.35	2.15
2417	cholesterol	117	13	27	1.99	0.55	2.54
2413		118	14	25	0.54	1.83	2.37
2411		185	18	29	0.09	1.22	1.31
Mean ± SD		132 ± 36	–	22.4 ± 13.1	1.01 ± 2.84	1.02 ± 2.16	2.03 ± 3.46
2709	High	157	10	65	0.0	3.11	3.11
4786	cholesterol	183	10	57	0.71	8.22	8.93
1640		225	14	225	1.08	11.34	12.42
502		236	10	144	1.34	9.21	10.55
Mean ± SD		200 ± 37	–	123.6 ± 168.4	0.78 ± 1.04	7.98 ± 9.27	8.77 ± 9.80
Level of significance		$P<0.05$	–	$P<0.001$	N.S.	$P<0.001$	$P<0.001$

[a] Monocytes attached to the luminal endothelial surface

* Cells in the SES – monocytes, macrophages and macrophage-foam cells/1,000 μ section length

** Total intimal cells, as in *. Sum of surface and SES cells

cells, macrophages and macrophage-foam cells were enumerated at 1000 × on the endothelial surface, and in the subendothelial space. Section length and intimal thickness were measured in microns with the aid of calibrated grids, and cell counts were expressed as the number of cells/1,000 μ section length. It can be seen (Table 1) that mean intimal thickness in the cholesterol-fat-fed animals was some 5-fold greater ($P>0.001$) than in the chow-fed animals, a difference reflecting the frequent presence of both fatty streaks and fibro-fatty plaques in the former. Although the number of monocytes attached to the luminal aspect of the endothelium did not differ significantly between the dietary groups, the cellularity of the subendothelial space (SES) was 8-fold greater in the cholesterol-fat-fed animals ($P<0.001$). The SES cells comprised mainly macrophages and macrophage-foam cells, with lesser numbers of monocytes. The greater cellularity of the SES in cholesterol-fat-fed animals is reflected in the significantly greater ($P<0.001$) intimal cellularity. These differences subsequently reflected the greater ease with which monocytes, macrophages, and macrophage-foam cells were found in the intima of the cholesterol-fat-fed animals ultrastructurally.

Ultrastructure. A total of 16 animals was studied ultrastructurally. Mononuclear cells were seen by transmission electron microscopy (TEM) either ad-

hering to or in close apposition to the aortic endothelium in both chow and cholesterol-fat-fed animals (Figs. 1 and 2). These surface cells exhibit many of the features traditionally associated with blood-derived monocytes: a relatively large indented or reniform nucleus, variable numbers of electron dense granules often located near the nuclear indentation, a prominent Golgi apparatus, numerous cisternae in the endoplasmic reticulum, and frequent villous cytoplasmic processes of variable length. Some processes appear to penetrate indentations on the endothelial cells, as seen in Fig. 1, where a villous projection is closely applied to the luminal aspect of an intercellular junction. Frequently, surface mononuclear cells are associated with relatively thin segments of endothelium (Fig. 2). The cell illustrated also overlies an intercellular junction.

The presence of mononuclear cells adhering to the aortic endothelial surface was confirmed by scanning electron microscopy (SEM), as illustrated in Figs. 3–7. A mononuclear cell derived from a chow-fed animal exhibiting short microvilli and ruffles is seen in Fig. 3. In Fig. 4, also from a chow-fed baboon, a relatively featureless leukocyte is seen either entering or leaving the SES. Adherent mononuclear cells were found more frequently on the aortas of cholesterol-fat-fed animals, with a distinct tendency to occur in clusters or aggregates over morphologically intact (non-denuded) endothelium as shown in Fig. 5. These cells vary in their appearance, some with numerous microvilli and others with few. Frequently, villous projections can be seen extending over and into the endothelium (Figs. 3, 5, 6 and 7).

Mononuclear leukocytes were observed in varying stages of penetration of the aortic endothelium. In Fig. 8, from a chow-fed baboon, part of a leukocyte is interposed between segments of essentially intact though relatively thin endothelium. The margins are not fragmented and the membranes appear intact, suggesting that this cell is moving between cells rather than through the endothelium. A short section of an intercellular junction adjoins the point of penetration. The leukocyte granules exhibit no discernible polarity. Figures 9 and 10 illustrate mononuclear cell penetration of the aortic endothelium in cholesterol-fat-fed baboons. In each instance a large cytoplasmic projection is interposed between segments of apparently normal endothelium, completely separating the endothelium and extending into the SES. As in Fig. 8, the endothelial cellular margins do not appear fragmented. SEM has confirmed these findings, and reveals leukocytes in comparable stages of interposition. Figure 4, the presumptive SEM equivalent of Fig. 8, shows a portion of a leukocyte projecting from the luminal aspect of the aortic endothelium. The endothelial margins and the surrounding endothelium are unremarkable. Electron micrographs give no clear indication of direction of movement. Although the surrounding endothelial margins have an everted disposition, it remains uncertain whether the two cells illustrated in Fig. 11 are entering or leaving the aortic SES.

Thus far we have described the scanning and transmission electron microscopic appearances of mononuclear cells adhering to the aortic endothelial surface, or interposed between segments of apparently intact endothelium. Figure 12, from a chow-fed animal, illustrates a mononuclear cell with sever-

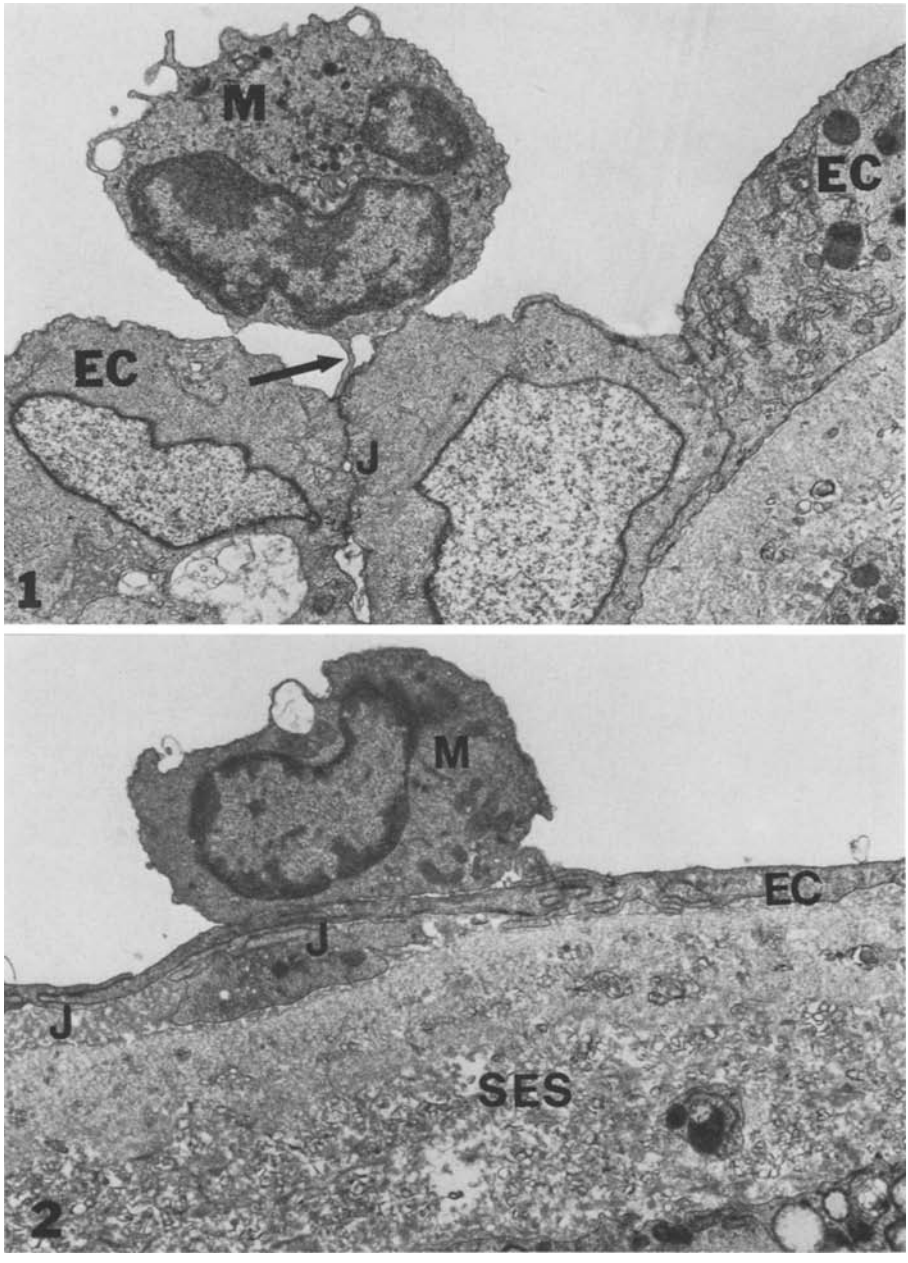


Fig. 1. Cholesterol-fat-fed baboon. Leukocyte with the morphologic characteristics of a monocyte (*M*) on intact aortic endothelium (*EC*), in proximity to an underlying junction (*J*). Note the cytoplasmic villous process (*arrowed*) projecting towards the junction (× 85,000)

Fig. 2. Monocyte (*M*) closely applied to thinned but intact aortic endothelium (*EC*) of a cholesterol-fat-fed baboon; note proximity to an underlying attenuated endothelial junction (*J*), and the prominent thickened subendothelial space (*SES*) containing floccular material and considerable extracellular myelin forms (× 85,000)

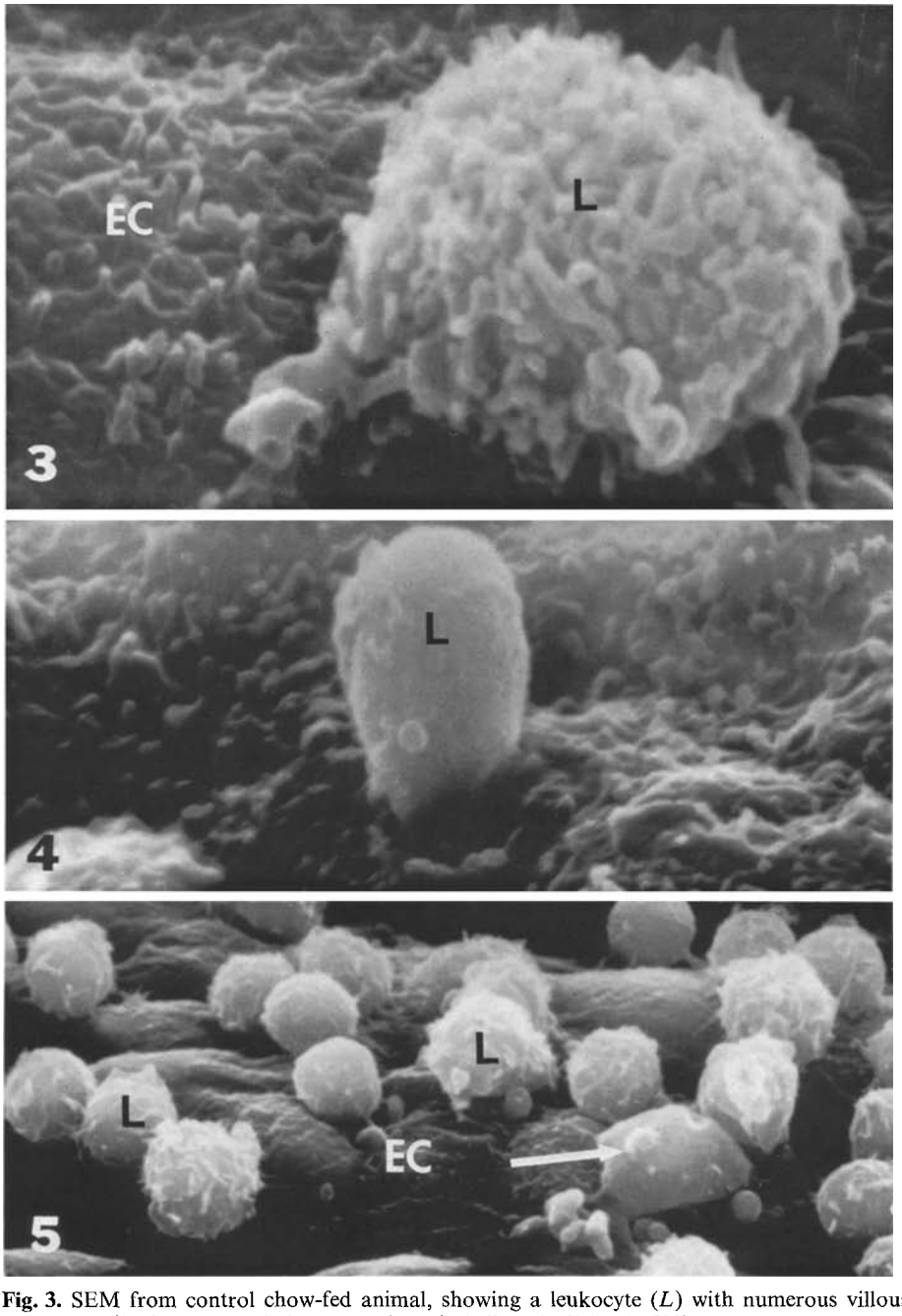
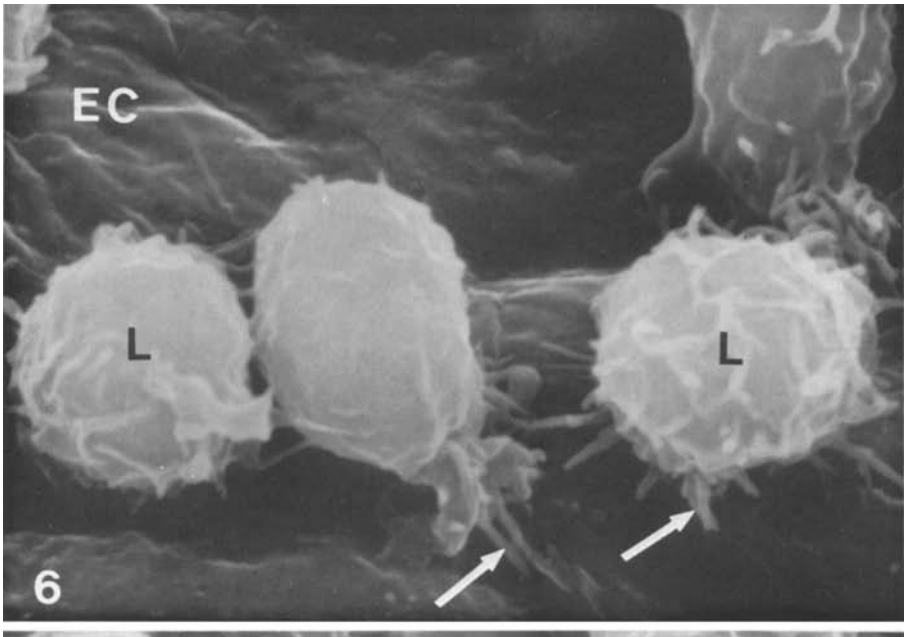


Fig. 3. SEM from control chow-fed animal, showing a leukocyte (*L*) with numerous villous processes adhering to overtly intact aortic endothelium (*EC*) (× 15,500)

Fig. 4. SEM from control chow-fed baboon, showing a leukocyte (*L*) either entering or leaving the aortic intima (× 8,500)

Fig. 5. SEM from a cholesterol-fat-fed baboon showing a cluster of leukocytes (*L*) adhering to or indenting the overtly intact aortic endothelium (*EC*). Occasional platelets are attached to the leukocytes (*arrowed*) (× 2,800)



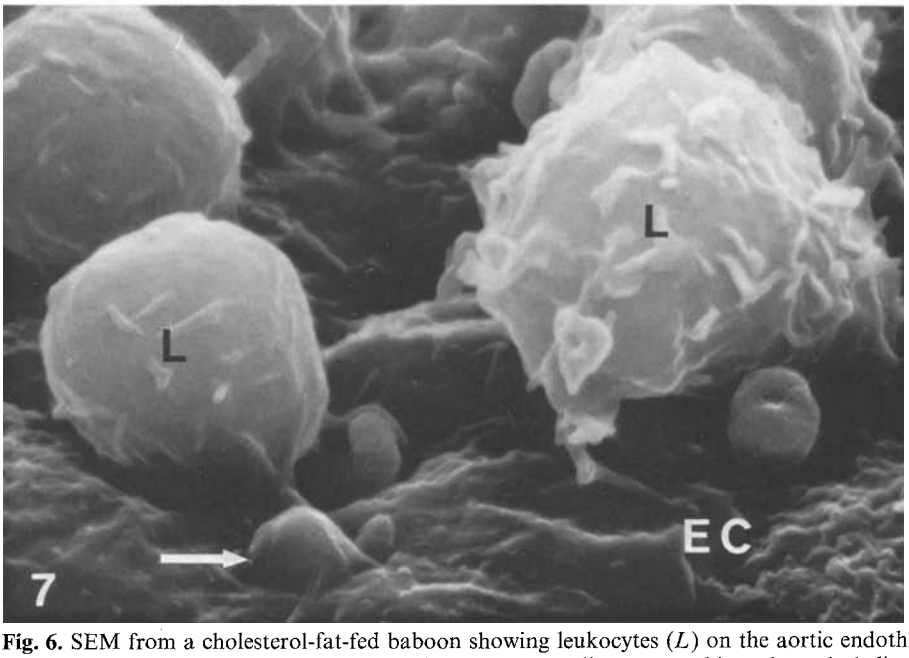


Fig. 6. SEM from a cholesterol-fat-fed baboon showing leukocytes (*L*) on the aortic endothelium (*EC*). Cytoplasmic processes (*arrowed*) are seen spreading over and into the endothelium. (×7,800)

Fig. 7. SEM from a cholesterol-fat-fed baboon showing leukocytes (*L*) on the aortic endothelium (*EC*). The cell to the left lies within a surface indentation, and another below it (arrowed) appears to be partially embedded within the intima (×6,000)

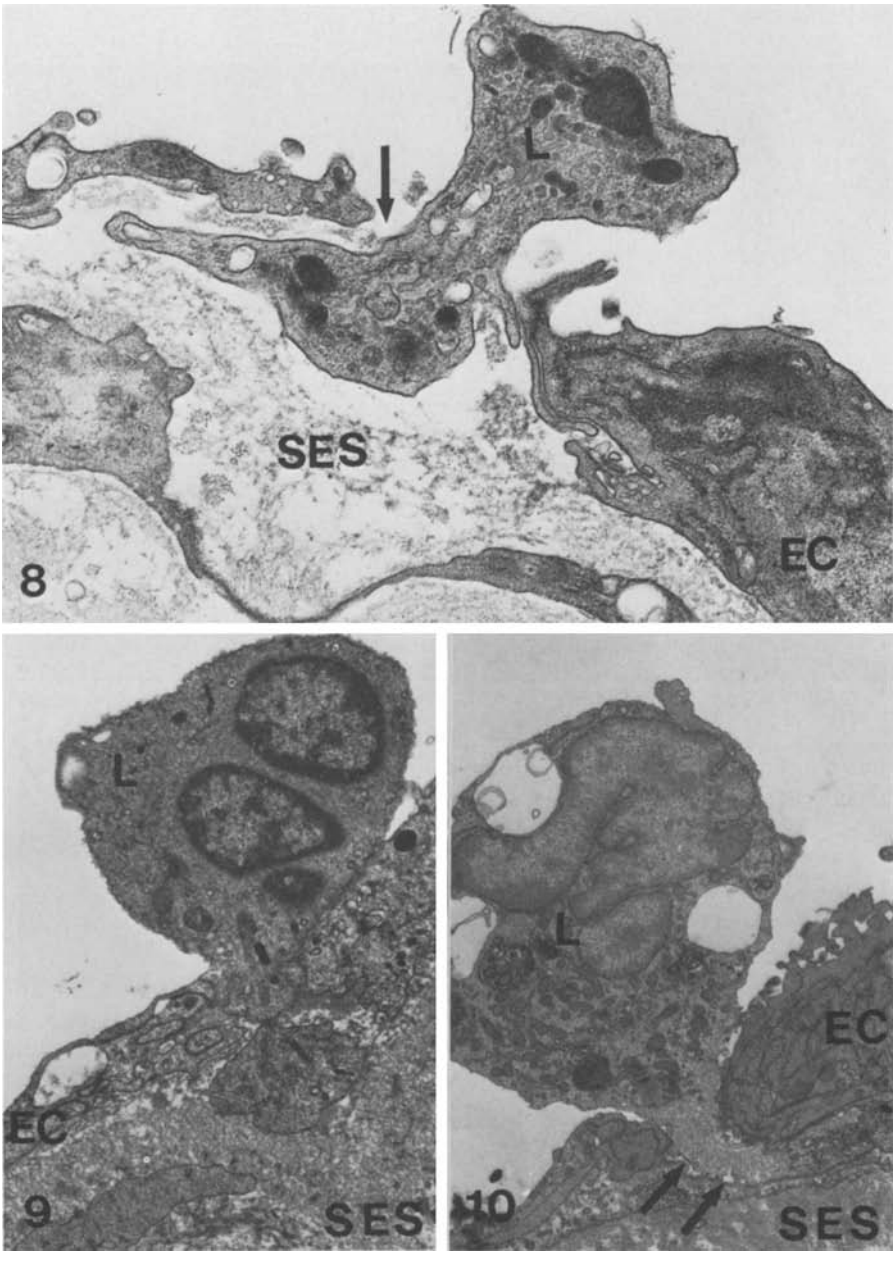


Fig. 8. Control chow-fed baboon. Portion of a leukocyte (*L*) is seen partly within the aortic lumen and the subendothelial space (*SES*). The membranes of adjoining endothelial cells appear intact. The endothelium (*EC*) is noticeably thinned. Note discontinuity in the endothelium (*arrowed*) (× 29,700).

Fig. 9. Portion of a leukocyte (*L*) protruding through thinned aortic endothelium (*EC*) to the subendothelial space (*SES*) in a cholesterol-fat-fed baboon (× 9,200)

Fig. 10. Elongate process (arrowed) of a leukocyte (*L*) with several vacuolar inclusions protruding through the aortic endothelium (EC) of a cholesterol-fat-fed baboon to the subendothelial space (*SES*) (× 6,800)

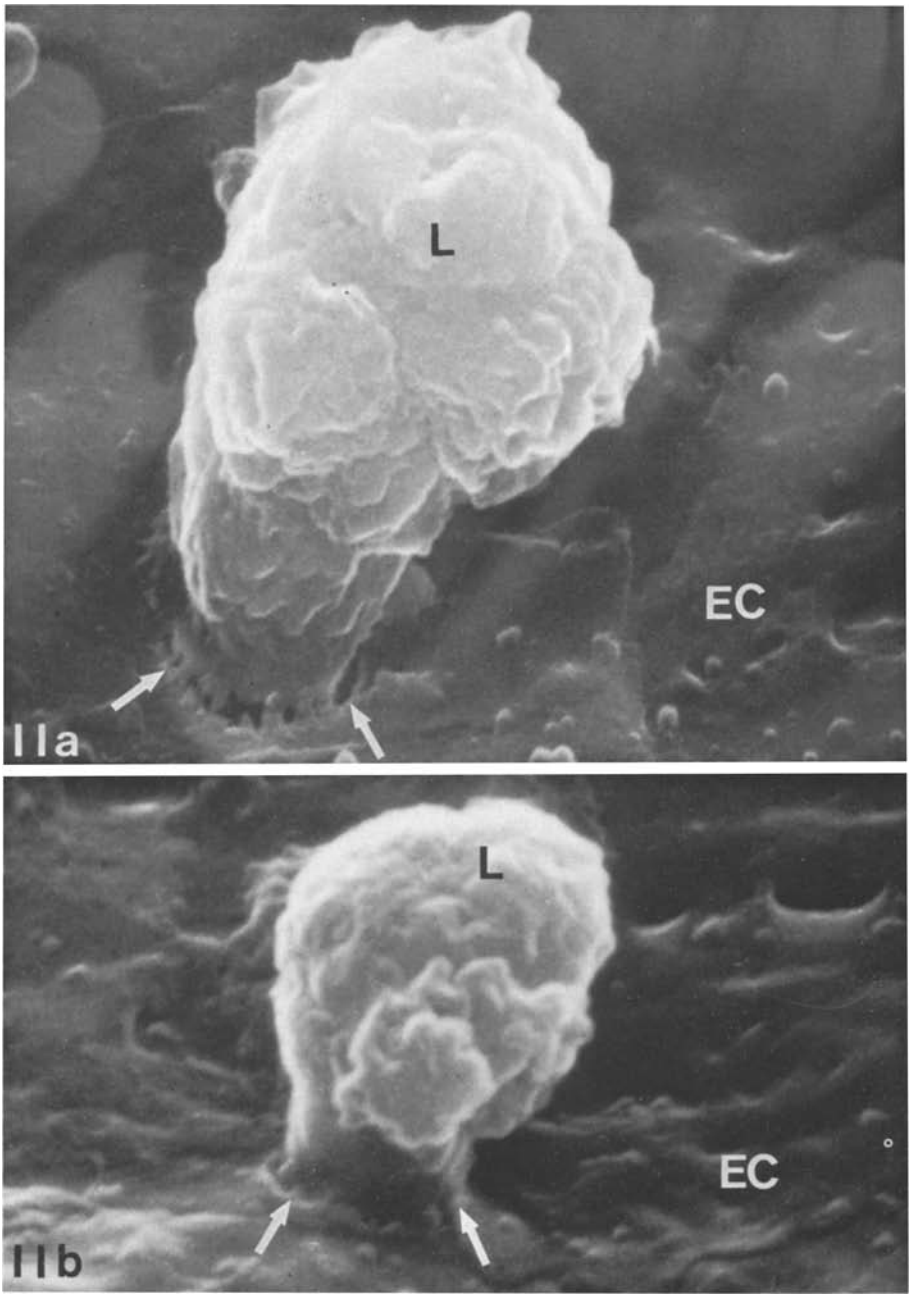


Fig. 11. SEM from a chow-fed animal. Upper and lower panels each show a (leukocyte) (*L*) partially embedded within the aortic endothelium (*EC*). Note the everted margins of the endothelium (*arrowed*). These cells are either entering or leaving the intima. (*Upper* ×22,500; *lower*, ×18,500)

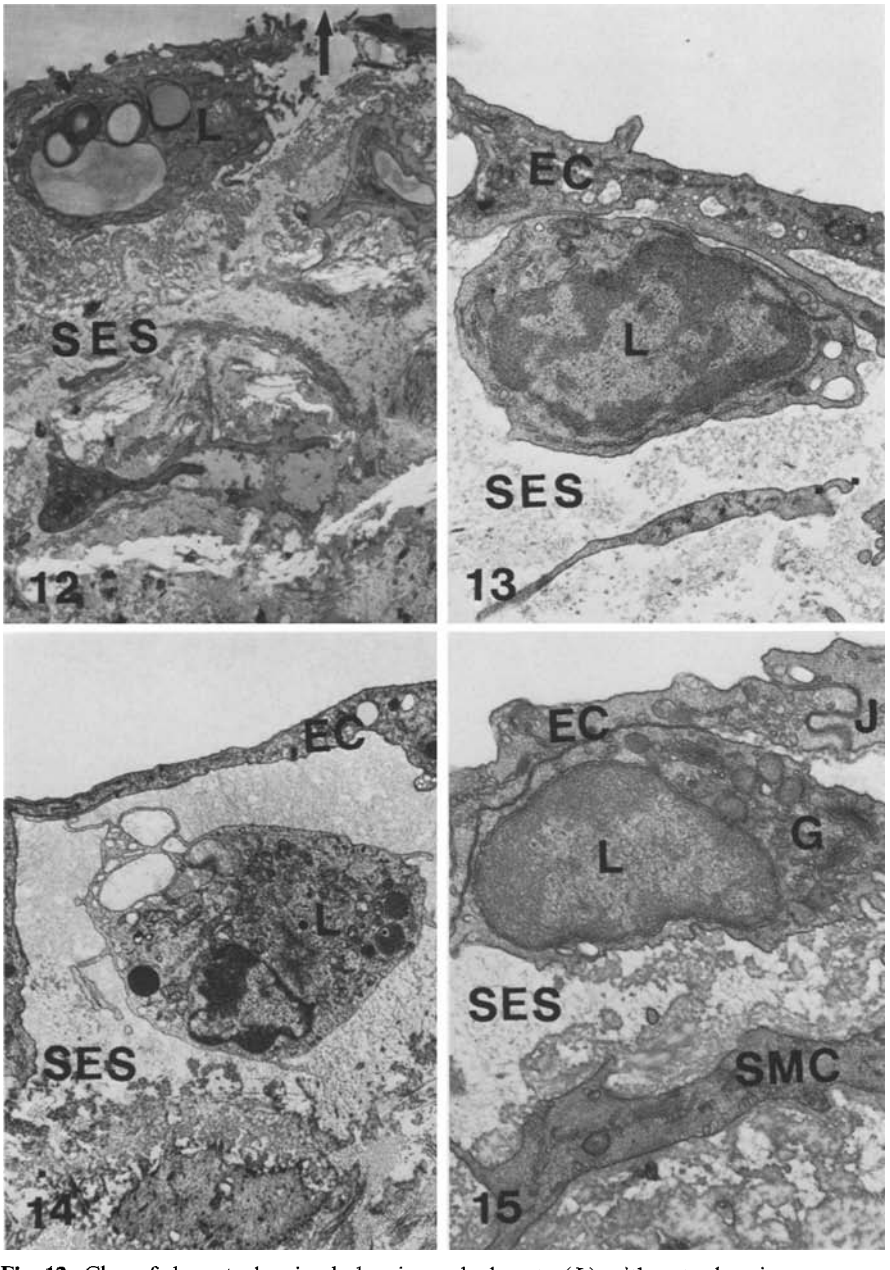


Fig. 12. Chow-fed control animal showing a leukocyte (*L*) with cytoplasmic processes and a number of translucent inclusions within the SES. The cell is closely applied to the overlying thinned endothelium. An irregular defect in the endothelium nearby is *arrowed* (× 6,500)

Fig. 13. Chow-fed control showing a leukocyte (*L*) in the subendothelium (*SES*) closely applied to the abluminal surface of the relatively thin endothelium (*EC*) (× 17,200)

Fig. 14. Chow-fed control showing a leukocyte (*L*) with some of the features of a macrophage within the widened floccular SES, exhibiting occasional villous projections, dense granules and some membrane-bound vacuolar inclusions. The overlying endothelium (*EC*) is attenuated but intact (× 5,100)

Fig. 15. Chow-fed control showing a leukocyte (*L*) making close contact with the thinned but intact overlying endothelium (*EC*) in which a junction (*J*) is apparent. Golgi profiles (*G*) are prominent. Part of a smooth muscle cell (*SMC*) is present within the subendothelium (*SES*) (× 5,600)

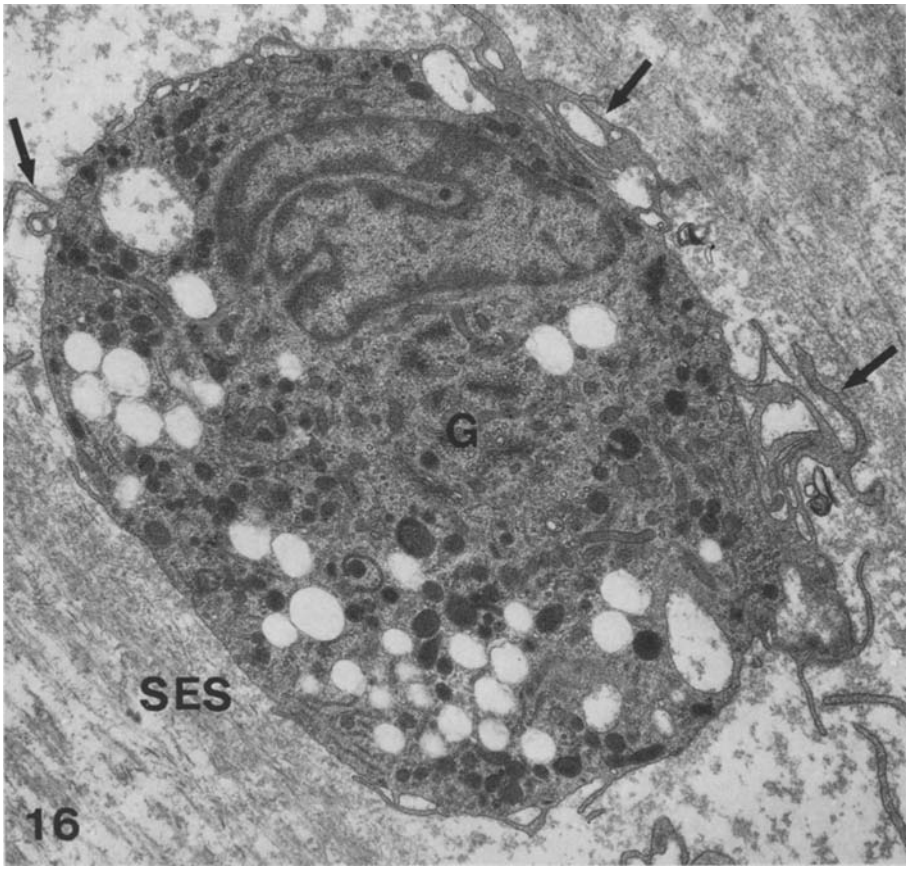


Fig. 16. Cholesterol-fat-fed baboon, illustrating an aortic intimal monocyte-derived macrophage within the SES containing both membrane-bound and non-membrane bound electron translucent lipid inclusions and numerous dense lysosomal granules. The Golgi apparatus (*G*) is readily seen. Villous cytoplasmic processes (*arrowed*) are present. (×9,000)

al large lipid vacuoles within the superficial SES adjoining a small defect or discontinuity in the relatively thin overlying aortic endothelium. The cell, which is surrounded by a floccular extracellular matrix, exhibits a number of cytoplasmic villous projections. Additional mononuclear cells within the SES, but unassociated with any endothelial defects in the plane of sections, are illustrated in Figs. 13–15. Frequently, these cells lie in close juxta-position to the abluminal surface of the endothelium (Fig. 13 and 15), which is typically thinned and attenuated.

While many mononuclear cells in the SES exhibit the traditional morphology of peripheral blood monocytes (Figs. 14 and 15), others show a range of appearances consistent with their transformation into macrophages (Figs. 12 and 16). This transitional sequence includes increasing cell size, the development of variable numbers of microvilli (Figs. 12 and 16), and numerous cytoplasmic organelles associated with a heightened secretory and

digestive function. The latter are reflected in the prominent perinuclear Golgi profiles, numerous lysosomes and residual bodies, and a copious endoplasmic reticulum with dilated cisternae. Membrane bound lipid inclusions can be seen in both chow-fed (Figs. 12 and 14) and cholesterol-fat-fed animals (Fig. 16), showing variable degrees of osmiophilia. Additionally, with increasing maturity, monocyte-derived macrophages not infrequently exhibit some peripheral margination of the nuclear heterochromatin (Fig. 16).

Discussion

We have demonstrated leukocytes showing the morphological characteristics of peripheral blood monocytes both on the endothelial surface and within the aortic subendothelial space (SES) of the chow-fed baboon (*Papio cynocephalus*). Other blood-derived cells, notably polymorphonuclear leukocytes, lymphocytes and eosinophils, were also observed in the normal baboon aortic intima but in appreciably smaller numbers. Monocytes and/or lymphocytes have been reported in the normal aortic intima of a number of species including rat (Joris et al. 1979), pig (Lee et al. 1970; Schwartz et al. 1980b) and man (Geer 1965). On the basis of their findings in the rat, Joris et al. (1979) have suggested that the presence of monocytes and lymphocytes in the normal aortic intima reflects a pathological response to a chemical message originating in the subjacent media, an interpretation with which we concur, particularly in view of our recent findings (Jauchem et al. 1981 and 1982, Valente et al. 1984) that cultured baboon aortic medial smooth muscle cells synthesize and release a chemoattractant for peripheral blood monocytes.

Mononuclear-macrophage recruitment to the arterial intima was enhanced approximately 8-fold in mildly hypercholesterolemic baboons receiving a cholesterol-fat supplemented ration relative to normocholesterolemic control animals receiving a chow diet alone (Table 1). Consistent with our present findings in the baboon is the enhanced monocyte recruitment to the arterial intima reported during hypercholesterolemia and atherogenesis in a variety of species including rabbit (Duff et al. 1957; Poole and Florey 1958; Hansson 1980), pig (Gerrity et al. 1979, Gerrity 1981), rat (Still and O'Neal 1962; Joris et al. 1983), rhesus monkey (Stary 1973; Schaffner et al. 1980), cynomolgus macaques (Stary and Malinow 1982), and man (Geer 1965; Baba et al. 1977).

Mechanisms responsible for monocyte recruitment to the normal intima, the enhanced recruitment in hyperlipidemic animals, and the implications of this phenomenon in atherogenesis are of particular interest. The attachment and subsequent migration of peripheral blood monocytes to the arterial intima in all likelihood involve at least four processes. Initially, circulating blood monocytes must be able to make contact with the endothelium, a process which is most likely to occur in areas of disturbed blood flow. Secondly, having made contact with the endothelium, the monocytes must then be able to attach. Implicit in this attachment phase, are changes in either the surface properties of the endothelium, the monocytes, or both.

Thirdly, directed movement through the endothelium implies that the cells must be capable of locomotion, and finally, because there is a net movement of cells to the SES, one may reasonably assume the presence of appropriate chemotactic gradients across the intima, which are recognizable by the adherent monocytes.

Attachment

A number of factors including platelets (Musson et al. 1979), cell surface proteins and charge (Hoover et al. 1978; MacGregor et al. 1978; Edelman 1976), and chemoattractants may modulate monocyte attachment to the endothelium. Platelets were rarely seen on the endothelial surface in either chow or cholesterol-fat-fed animals, making it unlikely that they are directly responsible for the monocyte attachment. However, as platelet aggregation or adhesion may be transient or evanescent, their role remains uncertain. In terms of sialoproteins and surface charge, it is of interest that dietary-induced hyperlipidemia in the rabbit, rat and pig (Weber et al. 1973; Balint et al. 1974; Gerrity et al. 1979), is associated with a thinning of the arterial endothelial glycocalyx. This change may be associated with qualitative or quantitative modification of surface glycoproteins and may contribute to the enhanced monocyte-endothelial attachment seen in animals receiving a cholesterol-fat supplemented diet.

Attachment of both neutrophils and peripheral blood monocytes to plastic surfaces is stimulated by chemoattractants such as C5a and the synthetic peptide n-formyl-methionyl-leucyl-phenylalanine (O'Flaherty et al. 1978). It is conceivable that chemoattractants originating within the arterial intima or media may modify the endothelial surface and enhance monocyte attachment, an effect which may be mediated through modulation of charge on the surface-membrane complex (Gallin 1980).

Transendothelial migration

In acute inflammation, migration of leukocytes across the vascular endothelium occurs via interendothelial junctions (Marchesi and Florey 1960). Our findings in continuous arterial endothelium indicate that most monocytes also appear to traverse the endothelium via interendothelial junctions. However, the occasional instance where a monocyte appears to have passed through rather than between the endothelial cells (Fig. 12) leaves this question open. In a number of instances defects in the endothelium were observed in close proximity to sites of cellular migration (Figs. 8 and 12). Such defects associated with leukocyte migration might disturb the normal endothelial permeability barrier function.

The direction of monocyte movement could not be deduced from the electron micrographs on the basis of monocyte polarity or the disposition of the endothelial cell margins. The uropod, described for lymphocytes in transit through high lymphatic venular endothelium (Schoefl 1972), which may indicate direction of movement (Norberg et al. 1973), was not a feature

of monocyte migration in this study. Neutrophils during oriented (Gallin 1980; Malech et al. 1977) migration on chemotactic membranes exhibit a polar redistribution of organelles, an alteration which was not observed in the monocytes in the present study.

Arterial wall chemoattractants

It is likely that both the attachment of monocytes to the arterial endothelium, and their subsequent migration to the SES, may be modulated by chemoattractants originating within the intima or media. This concept is supported by the observation that leukocyte-endothelial adhesion *in vitro* is enhanced by chemotactic agents (Hoover et al. 1980). A number of biological agents have been shown to possess chemoattractant activity for monocytes and/or neutrophils, including fibroblast (Sobel and Gallin 1979) and smooth muscle cell-derived factors (Jauchem et al. 1981 and 1982; Valente et al. 1984), collagen and its constituent chains (Postlethwaite and Kang 1976), elastin-derived peptides (Senior et al. 1980), and HETE, an hydroxy fatty acid lipoxygenase derivative of arachidonic acid (Turner et al. 1975; Goetzl 1976). Membrane lipid synthesis and composition have also been shown to influence leukocyte chemotaxis (Pike and Snyderman 1980), indicating a broad spectrum of interactions and the need for further explorations in this area.

Role of monocytes in atherogenesis

The early stages of atherogenesis are characterized by an enhanced vascular leakage, resulting in the intimal accumulation of plasma constituents (Schwartz et al. 1978) and the appearance of blood-derived cells, notably monocytes, lymphocytes and the occasional neutrophil and eosinophil. When the disease has reached an advanced stage, adventitial lymphocytic infiltration is present in approximately 80% of lesions (Schwartz and Mitchell 1962). Along with Joris and Majno (1979) we consider that atherosclerosis fulfills the contemporary criteria defining an inflammatory process in which the peripheral blood monocyte is an important participant. One role is their scavenger or phagocytic function (Bainton 1980), and another as progenitors of at least part of the macrophage-foam cell population of the plaques. This latter is consonant with our present observations of transitions from the typical morphology of the peripheral blood monocyte (Nichols and Bainton 1975) to the foam cell-macrophage characteristic of the atheromatous intima. Transformation of monocytes/macrophages into distinct foam cells has also been described extravascularly in granulomata (Schwartz et al. 1984). In evolving granulomata, mitoses in macrophages were infrequent (Schwartz et al. 1984). This finding is consistent with the carrageenan granuloma as a low turnover lesion, in which the constituent macrophages have a long life and are augmented by a low rate of cell division (Ryan and Spector 1969). Mitotic figures were not observed in intimal macrophages and foam cells in the present study, in contrast to

their presence in other species of nonhuman primates (Stary and Malinow 1982). Overall, it appears unlikely that macrophage replication contributes significantly to the intimal foam cell population.

Other roles of the blood monocyte in atherogenesis involve their immunologic function as processors of antigen and in lymphocyte activation (Pierce 1980), and their secretory function with the release of a spectrum of hydrolytic enzymes including elastase and collagenase (Unanue 1976; Davies and Bonney 1979), and lipoprotein lipase (Khoo et al. 1981).

Because of the importance of smooth muscle cell proliferation in the pathogenesis of the atheromatous plague, the mitogenic potential of the monocyte-macrophage is of particular importance. Macrophages have been shown to stimulate the proliferation of both fibroblasts and vascular smooth muscle cells in culture (Leibovich and Ross 1976; Greenburg and Hunt 1978). More recently Glenn and Ross (1981) have demonstrated that activated peripheral blood monocytes release a mitogen for vascular SMC, emphasizing another important link between the peripheral blood monocyte and atherogenesis.

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