Virchows Arch. A Path. Anat. and Histol. 365, 23—40 (1975)

Scanning Electron Microscopic Observations of Endothelial Changes in Experimentally Induced Atheromatosis of Rabbit Aortas

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Received June 1, 1974

Summary. Following the administration of cholesterol for a period of 6–7 weeks, Scanning Electron Microscopic (S.E.M.) observations revealed mono-cellular, crater-like and dome-shaped endothelial changes on top of the large intimal plaques in the rabbit aortas. Finger-like and other shaped cell protrusions were observed at the edges of these crater-like and dome-shaped endothelial changes, giving the intimal plaques a rough appearance. At other sites, normal, smooth, although irregularly arranged, endothelial cells covered the lesions. By impregnating the cell borders with silver-nitrate or silver proteinate containing perfusates, it was possible in most cases to ascertain that the lesions were derived from changes in one cell or from changes in a small collection of cells.

S.E.M.-observations further revealed crater-like and dome-shaped endothelial changes to be present in large fields or as isolated cell changes in normal areas at sites where no gross lesions were observed with the light microscope.

In addition large, multi-cellular, crater-like endothelial changes were observed at the edges of the large intimal plaques. At these sites several endothelial cells were lacking, leaving behind a crater in which sometimes cells and a few fibrin threads were found.

Following the administration of cholesterol for periods of 4–5 and 2–3 weeks similar mono-cellular changes were observed, some extending over large areas, others as single cells in apparently normal surroundings. Quantitatively the number of lesions was less than when the cholesterol was administered for a longer period. Transmission electron microscopic studies revealed the presence of large amounts of membrane-bound lipid globules in the subendothelial spaces and within some endothelial cells, structures which were assumed to be cross-sections of the crater-like or dome-shaped endothelial cell protrusions, visible with the S.E.M.

Key words: Aortic Diseases — Scanning Electron Microscopy — Biological Transport — Endothelial Cells.

Introduction

The recent introduction of the Scanning Electron Microscope (S.E.M.) as an analytical tool in the study of pathologically changed tissue has re-opened interest in the endothelial changes of experimentally induced or naturally occuring atheromatous aortic lesions (Shimamoto, 1969, 1971; Riede and Villigers, 1970; Garbarsch and Collatz Christensen, 1970; Smit and Ryan, 1971; Weber and Tosi, 1971a; Buss, 1973; Collatz Christensen, 1974).

However, the information so far obtained is restricted by the technical problems related to the cleaning and subsequent fixation of the endothelial surface and the visualization of individual endothelial cells.

Loss of surface structures was reported by Walton (1970) in the initial stages of atherosclerosis affecting aortic valves, and Buss (1973) mentioned variations in the number of endothelial cells in a given area. The normal pattern of endothelial

folds and gullies and the endothelial cell bridges were reported to undergo flattening and oedematous swelling following two weeks of cholesterol administration (Shimamoto *et al.*, 1969 and 1971). Four weeks administration of cholesterol resulted in the appearances of elevated lesions with highly deformed and swollen, degenerate areas. Garbarsch and Collatz Christensen (1970), and De Bruijn *et al.*, (1974), using perfusion techniques and cell border impregnation with silver nitrate or proteinate were unable, however, to detect cell bridges in normal aortas. The presence of a veil-like membrane over the plicae in the aorta was reported after 24 days of cholesterol administration in rabbits and very hypertrophied intimal plicae after 45 days by Weber and Tosi (1971b), and following longer cholesterol administration in guinea pigs (Weber and Tosi, 1971a). Calcification, fibrin, lipid and cholesterol deposits were observed by Riede and Villigers (1970) in atherosclerotic human aortic ulcers.

Because of the technical problems, interest in developing new techniques to demonstrate the endothelial cell borders have been revived. It was found that techniques already used for demonstration of endothelial cell borders by light microscopy, using silver nitrate impregnation, could be applied to the study of endothelial changes with the S.E.M. (Garbarsch and Collatz Christensen, 1970; Geissinger 1972). Recently the use of silver proteinate for impregnating endothelial cell borders has been advocated (De Bruijn *et al.*, 1973 and De Bruijn *et al.*, 1974).

This investigation reports the S.E.M. observations of endothelial changes due to experimentally induced atheromatosis in rabbit aortas following the administration for 6–7, 4–5 or 2–3 weeks of a cholesterol containing diet, using silver nitrate or silver proteinate to delineate endothelial cell-borders. The results were compared with Transmission Electron Microscopic T.E.M. observations of similarly treated rabbits.

Material and Methods

6–8 week old female rabbits (strain New Zealand) of about 3 kg weight were used for these experiments. The animals were fed pellet chow to which cholesterol was added to ensure a daily consumption of about 1g cholesterol. Water was given ad libitum. The cholesterol diet was maintained for 6–7 weeks, 4–5 weeks or 2–3 weeks respectively. At the end of each period the animals were anaesthesized by intramuscular Hypnorm (Philips-Duphar, Amsterdam) 1 ml per kg body weight. Subsequently the chest was opened and the aorta was freed from blood by perfusion with Dextran (Macrodex with glucose or Macrodex with NaCl; Poviet, Amsterdam) at a pressure of 1600 mm H_2O/cm^2 at 37° C for two minutes.

Cell border impregnation was obtained by perfusion with either $AgNO_3$, according to the methods of either Sinapius (1959) or Garbarsch and Collatz Christensen (1970), or with silver proteinate according to the method of De Bruijn *et al.* (1974) for 4 minutes at room temperature at the same pressure. Subsequently the aortas were carefully rinsed with saline at 37° C for 2 min and fixed with 3% glutaraldehyde solution in 0,1 M cacodylate buffer of pH 7.4, for 7 min at 5–10° C using the same pressure.

Following fixation in the same glutaraldehyde solution for 24 hrs, ring-shaped pieces were cut from the aortas, carefully rinsed in distilled water, frozen in liquid nitrogen and dried *in vacuo* at –70° C overnight. Subsequently these aortic rings were carefully opened and gold was evaporated on to the endothelial side.

The specimens were observed in a Jeol scanning electron microscope SM3 at 25 KV which was kindly placed at our disposal by Jeol, Europe. The preparations for observation with the T.E.M. are as described elsewhere (De Bruijn, 1973) and as used for a Philips E.M. 300 at 60 KV.

Results

I. Scanning Electron Microscopic Observations

Impregnation of the rabbit aortas with either silver nitrate, according to the methods of either Sinapius (1956) or Garbarsch and Collatz Christensen (1970), or following impregnation with silver proteinate (De Bruijn *et al.*, 1974) resulted generally in a clear delineation of the endothelial cell borders in normal animals. The unchanged endothelial cells of the aortas from rabbits fed a cholesterol containing diet were equally well demonstrated by all three methods. As a consequence, the results to be described are those found in animals treated by any of the three mentioned methods. Reasons for favouring the use of the silver proteinate method have been discussed by De Bruijn *et al.*, (1974).

Aortas from three different groups of animals were investigated as follows:

a) Following administration of a cholesterol-containing diet for 6–7 weeks.

b) Following administration of a cholesterol-containing diet for 4–5 weeks.

c) Following administration of a cholesterol-containing diet for 2–3 weeks.

a) Cholesterol-Containing Diet for 6–7 Weeks

S.E.M. observations revealed three different types of endothelial lesions in the aortas:

1) Clearly elevated areas such as the fatty spots and streaks

2) Damaged areas that did not bulge into the lumen

3) Damaged spots in apparently normal areas.

1. Fatty Spots Recognizable with the Light Microscope. These appeared in the S.E.M. as elongated ridges in almost flat endothelial surroundings (Fig. 1). In most cases the endothelial lining of these ridges consisted of evenly arranged endothelial cells, clearly outlined by silver impregnated lines. In some areas, however, the cell border was only faintly outlined or even absent. In some places at the top of these ridges, the endothelial lining exhibited changes. Finger-like or curtain-like filamentous structures protruded into the lumen thus forming funnel-shaped structures (Fig. 2). In some cases these endothelial changes were such, that only one or two cells were involved; such changes will be referred to henceforth as mono-

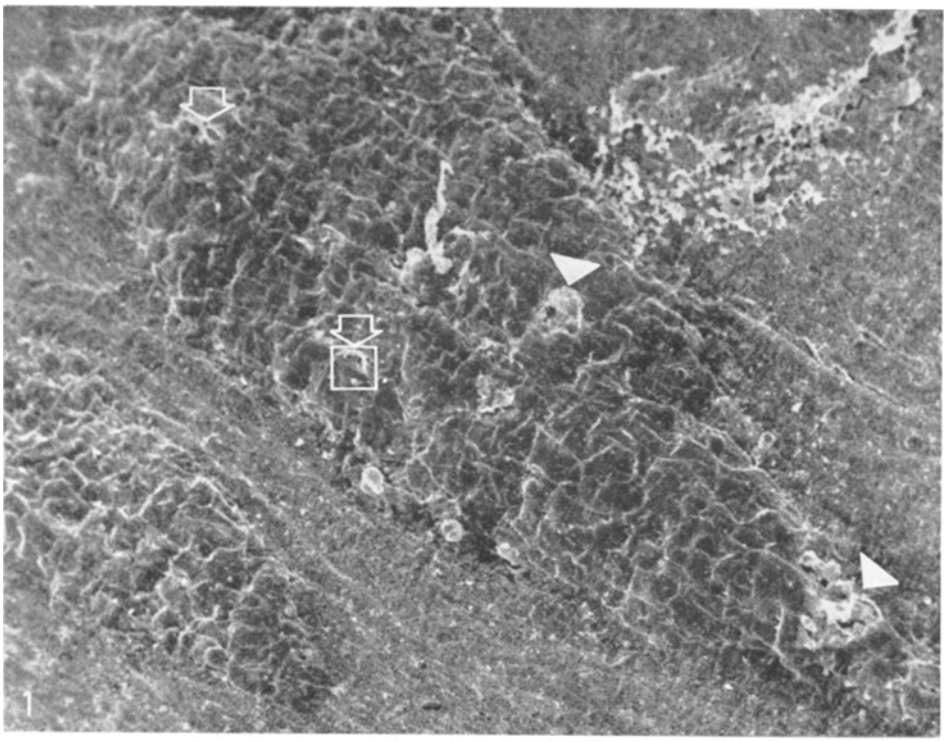
Figs. 1—10. Endothelial changes on large fatty streaks and spots in the S.E.M.

Fig. 1. Two elongated fatty streaks at low magnification. Cell border demarkation is just visible. The arrow () marks the mono-cellular changes, the arrowhead the multi-cellular changes (▼). (6 w. Cholesterol, Sinapius, M × 190)

Fig. 2. Detail of the mono-cellular endothelial change from Fig.1 at higher magnification. Finger-shaped cellular protrusions were present on funnel-shaped (), curtain-shaped () and more slit-shaped changes (bended arrow)

Fig. 3. Mono-cellular crater-like endothelial changes separated from each other on top of a fatty spot (arrows). The small arrowheads mark the cellborders. (6 w. cholesterol, Garbarsch, M × 750)

Fig. 4. Mono-cellular dome-shaped endothelial changes close to each other on top of a fatty spot. Some domes are open, others closed, giving the surface a cobblestone appearance ( ). (6 w. Cholesterol, Sinapius, M × 1350)



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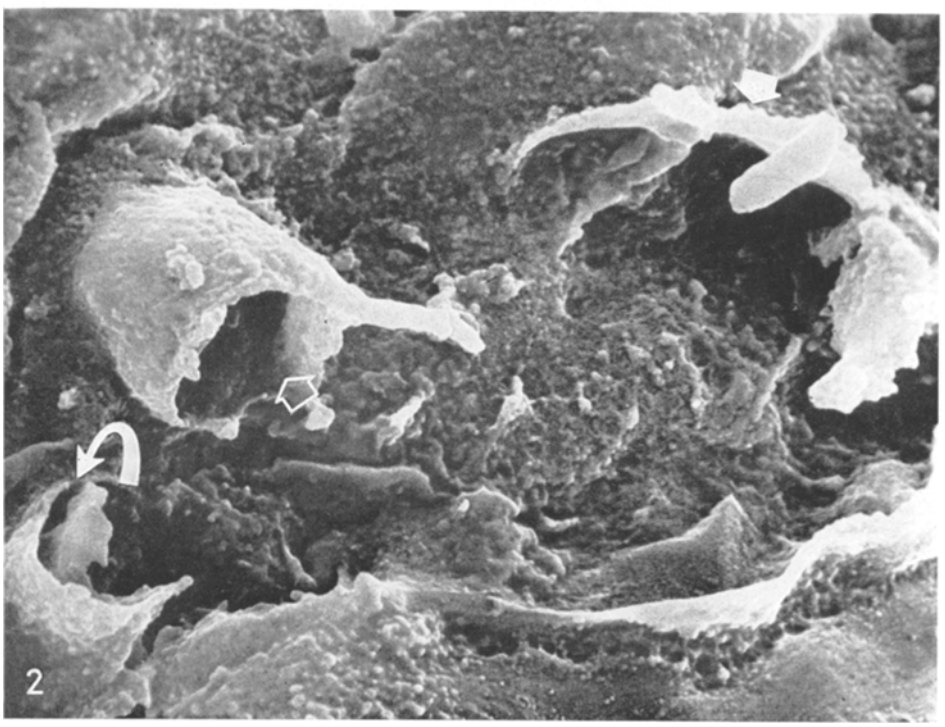

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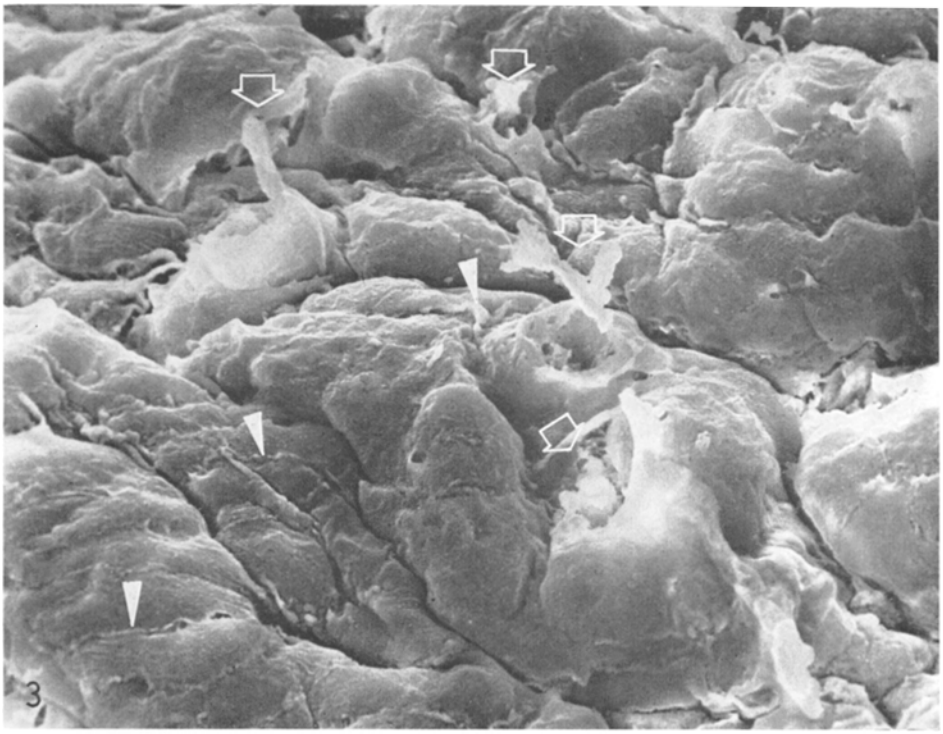
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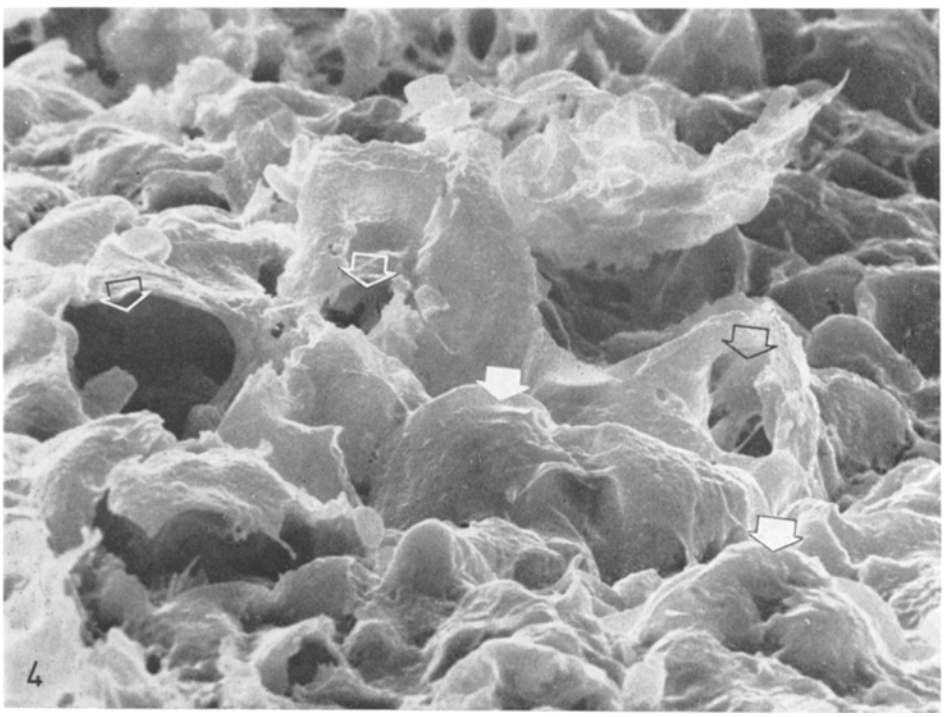
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cellular changes (Fig. 2). In other places several cells were involved, which will subsequently be referred as multi-cellular changes.

In Fig. 3 an area on top of a large fatty spot is shown, where at least four mono-cellular changes were present (◁▷) separated from each other by areas with rather faintly outlined, smooth surfaced endothelial cells. However, when such mono-cellular lesions were not separated from each other by smooth surfaced cells, whole areas became rough and irregular (Fig. 4).

The mono-cellular changes generally consisted of crater-like indentations in the endothelial surface. At the edges, cell protrusions were present which varied from thin delicate filaments (Figs. 2 and 7) to rather broad, robust pillars (Fig. 8), sometimes indented (Fig. 9) or with ribbon or helmet-like structures (Fig. 10). Sometimes the impression was gained that the cell protrusions formed lids, ready to close the boxes beneath (Fig. 11). Inside the crater-like indentations, the endothelial cell surface still appeared to be present, although several holes were observed inside the craters some in the assumed endothelial surface (Fig. 9), others in the underlying structure (Fig. 9 and Fig. 10). These mono-cellular changes were frequently observed on the slopes of the large atheromatous ridges at sites facing the bloodstream.

In the large multi-cellular type of change, short fibrillar structures were frequently seen inside the craters (Fig. 5). In some craters cells were present, sometimes erythrocytes, and sometimes rather large cells with multiple short pseudopodia (Fig. 6). These multi-cellular changes were more frequently observed at the edges of the large fatty spots than on top of them.

In some aortas in the neighbourhood of the large fatty spots visible with the light microscope, much smaller spots that clearly bulged into the lumen were revealed with the S.E.M. (Figs. 1 and 5). The endothelial linings of these smaller spots generally showed mono-cellular type of changes in addition to smooth surfaced endothelial cells (Fig. 5).

Another group of endothelial changes that were observed were the more dome-shaped mono-cellular endothelial changes. On top of the large intimal

Fig. 5. Large crater-like multi-cellular endothelial change at the margin of a fatty streak. Inside the crater fibrin threads were present, cellular protrusions are present at the crater edge (arrow). A small fatty spot is present in the lower half of the picture with mono-cellular crater-like changes (arrowheads). (6 w. Cholesterol, Garbarsch, M × 310)

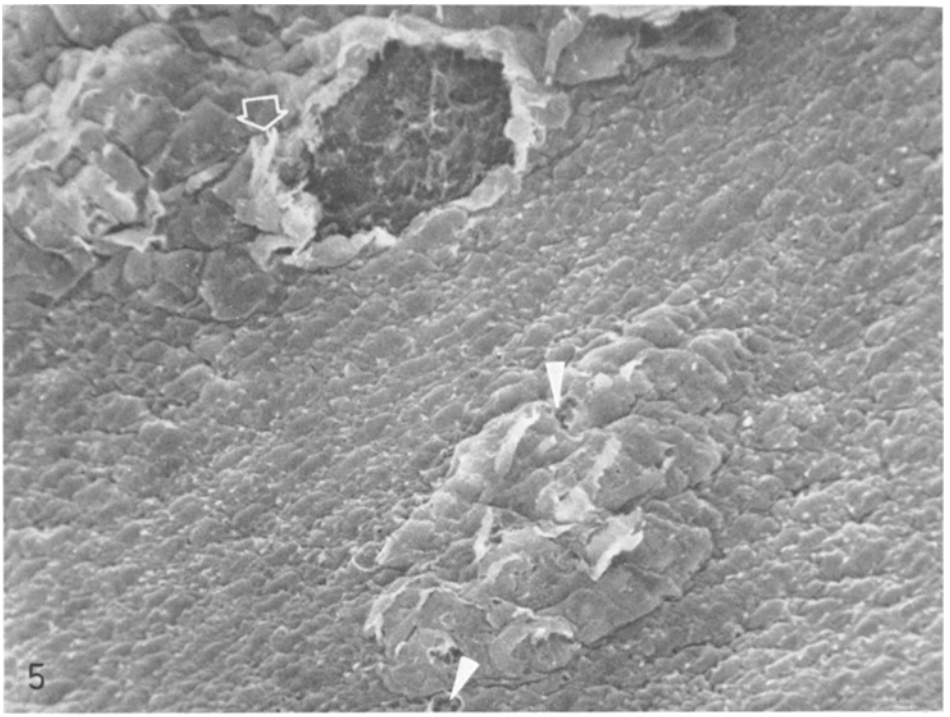
Fig. 6. In some of the multi-cellular changes cells were present. Apart from the trapped erythrocytes, cells were present with short pseudopods (arrow). (6 w. Cholesterol, Sinapius, M × 1450)

Fig. 7. Delicate finger-shaped protrusion at the mono-cellular crater-edge. Note the holes in the surface of the crater (arrow). (6 w. Cholesterol, Sinapius, M × 15600)

Fig. 8. Sturdy pillar at the edge of a crater-like indentation. (6 w. Cholesterol, Garbarsch, M × 3750). Detail from Fig. 3

Fig. 9. Dentated structure at the edge of a crater. Note the holes in the apparent cell surface ◁▷ and those in the more deeper parts of the crater. (◀▶) (6 w. Cholesterol, Garbarsch, M × 2500) Detail from Fig. 3

Fig. 10. Ribbon-like structures at the edge of the crater. (6 w. Cholesterol, Sinapius, M × 5050)



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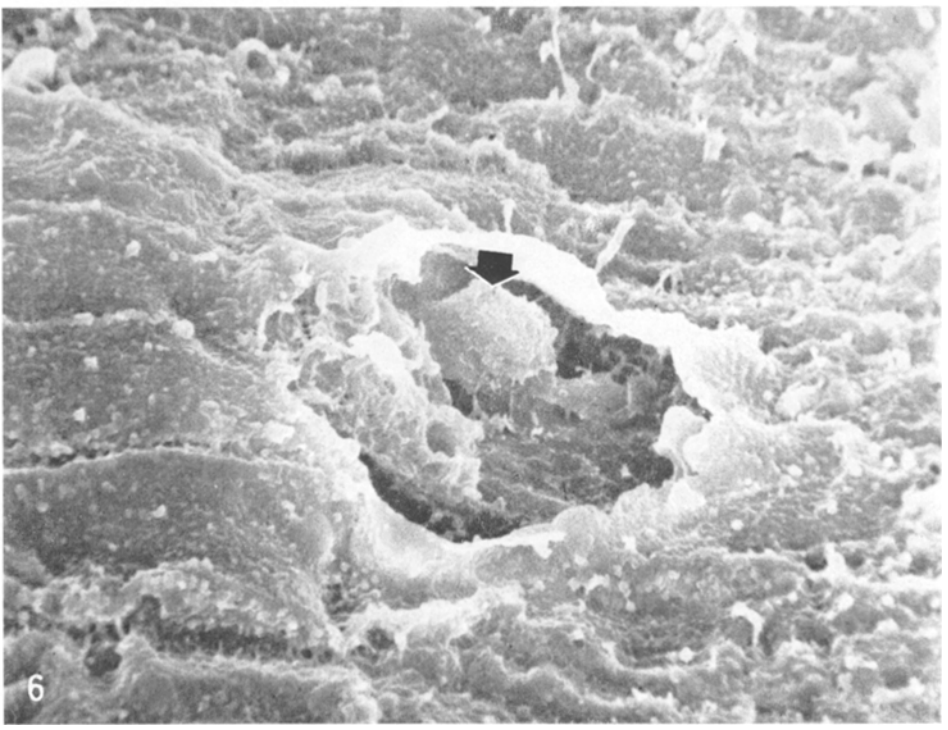

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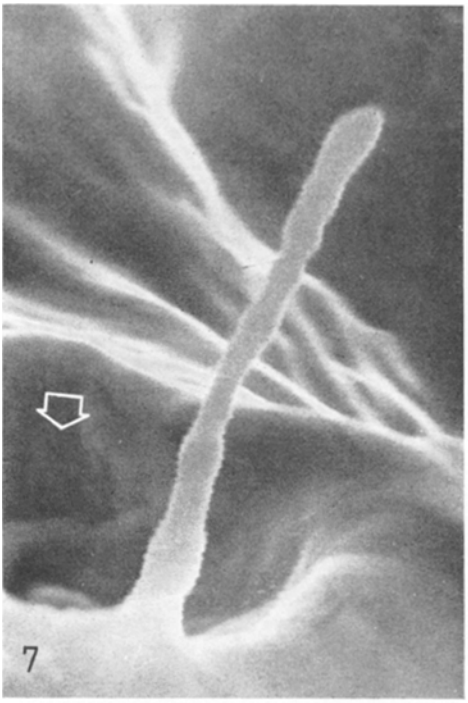
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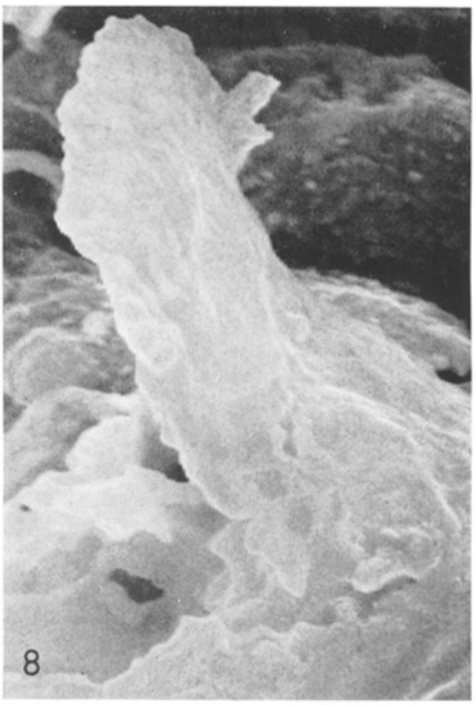
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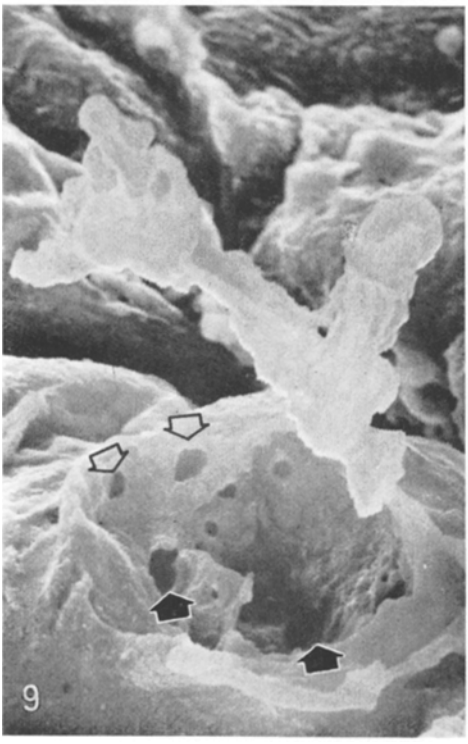
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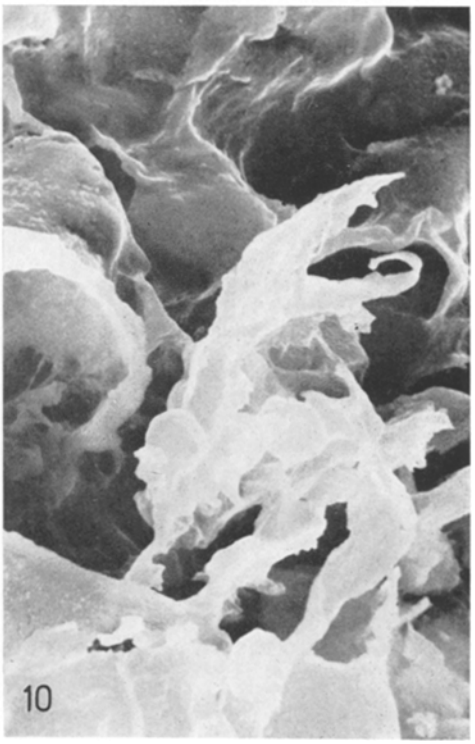
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plaques, areas containing dome-shaped mono-cellular changes gave the plaque a cobblestone appearance (Fig. 4 arrows). In some of these open domes no contents were observed, in others material was present. In some cases the contents of the domes had a sheet-like (crystal-like) appearance (Fig. 12). On some domes the cell protrusion at the margin had partly covered the contents; the edges of the cell protrusions being rounded and dull.

2. Damaged Areas that Did not Bulge into the Lumen. In addition to the areas with dome-shaped mono-cellular changes on top of the intimal plaques, similar large areas were observed that did not clearly bulge into the lumen (Figs. 11 and 12). These lesions were not observed with the light microscope.

3. Damaged Spots in Apparently Normal Areas. Endothelial changes observed in the third type of aortic lesions after 6–7 weeks of cholesterol administration were not so very different from the two types of lesions already described except that the changes were observed in apparently normal areas. Isolated dome-shaped and crater-like mono-cellular changes were observed with fibrin threads and erythrocytes trapped at the entrance (Fig. 13). It was difficult to decide whether the (cellular ?) surface inside the dome differed from that outside (Fig. 13), and whether the observed holes were present in the cell surface or between the cells. Isolated small multi-cellular changes were also observed (Fig. 14). Inside the small craters short-filamentous structures were seen. These were different from the assumed endothelial cell surfaces around the crater (Fig. 14, ▼ and ▽). A funnel-shaped appearance leading to the assumed sub-endothelial zone was also observed. Due to the cell border demarcation by silver proteinate the contribution of each cell to this process was clearly observed (Fig. 14 arrows).

b) Cholesterol-Containing Diet for 4–5 Weeks

The differences between the lesions in the aortas from rabbits fed a cholesterol diet for 4–5 weeks compared with those for 6 and 7 weeks were only of a quantitative and not qualitative nature. The number of light microscopically observed lesions was reduced, but the number of rather small lesions of type 2 and 3 ob-

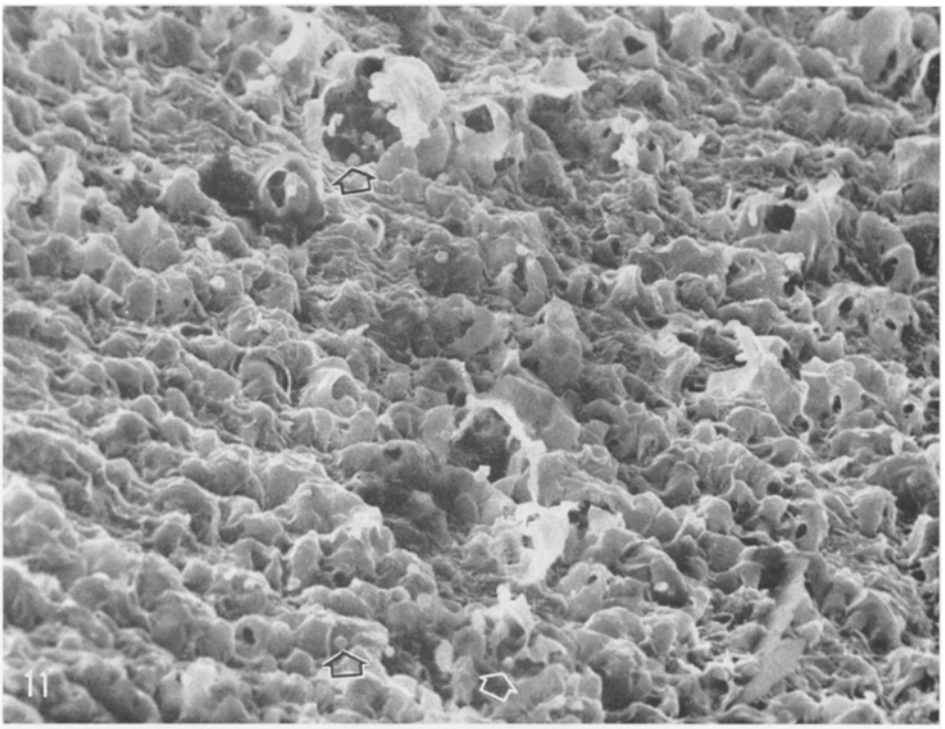
Figs. 11—14. Endothelial changes in areas not clearly bulging in the lumen. Note the holes inside the crater

Fig. 11. Large field of predominantly dome-shaped endothelial changes in an area which is not clearly bulging in the lumen. Some domes are closed, others are open with a great variety of hole diameter. Some crater-like changes with erythrocytes trapped in the crater are also present (arrow). (6 w. Cholesterol, Sinapius, M × 375)

Fig. 12. Dome-shaped endothelial changes with sheets of crystal-like material (arrows), only partly covered by the endothelial cell protrusion. There is some doubt whether one cell or a small group of cells are active here. (6 w. Cholesterol, silver proteinate, M × 4000)

Fig. 13. Single dome-shaped mono-cellular endothelial change. Fibrin threads have trapped an erythrocyte at the entrance. Inside the dome holes are discernable (arrows), but it is hard to judge whether the endothelial surface observed at the outside of the dome, which is continuous with the endothelial surface in the entrace of the dome is disrupted at these sites of the holes or not. (6 w. Cholesterol, Sinapius, M × 4400)

Fig. 14. Isolated small multi-cellular endothelial change. The arrows mark the cell borders decorated here by silver proteinate. The short-fibrillar surface ▼ inside the crater of these endothelial cell protrusions is clearly different from the cell surfaces ▽ at the outside of the crater. The funnel-shaped protrusion at one end (<>) leads into a hole which is assumed to be underneath the endothelial cell. (7 w. Cholesterol, De Bruijn, M × 2750)



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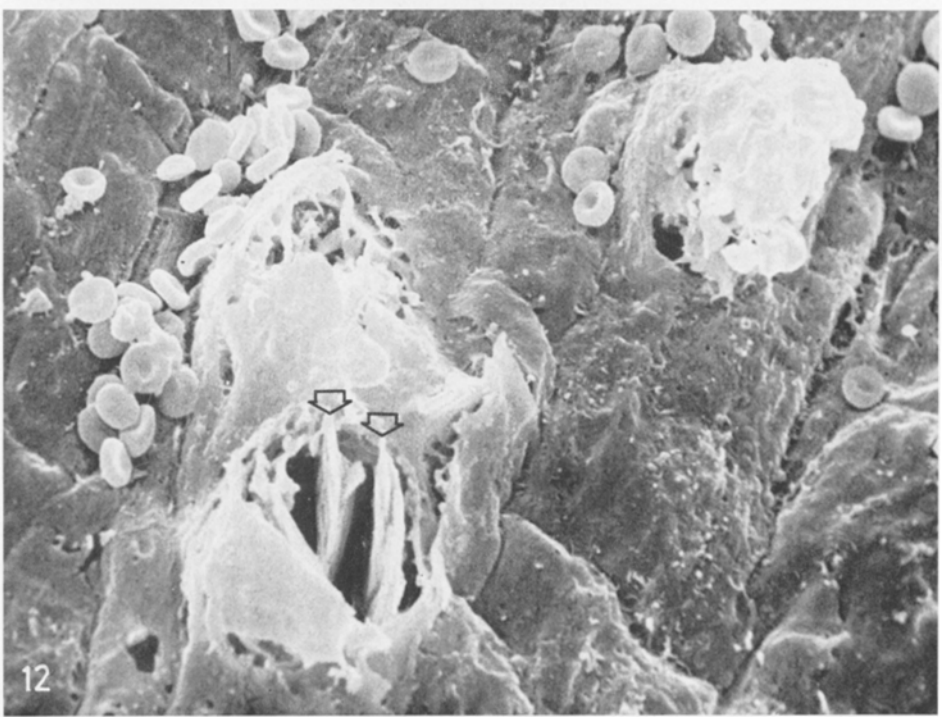

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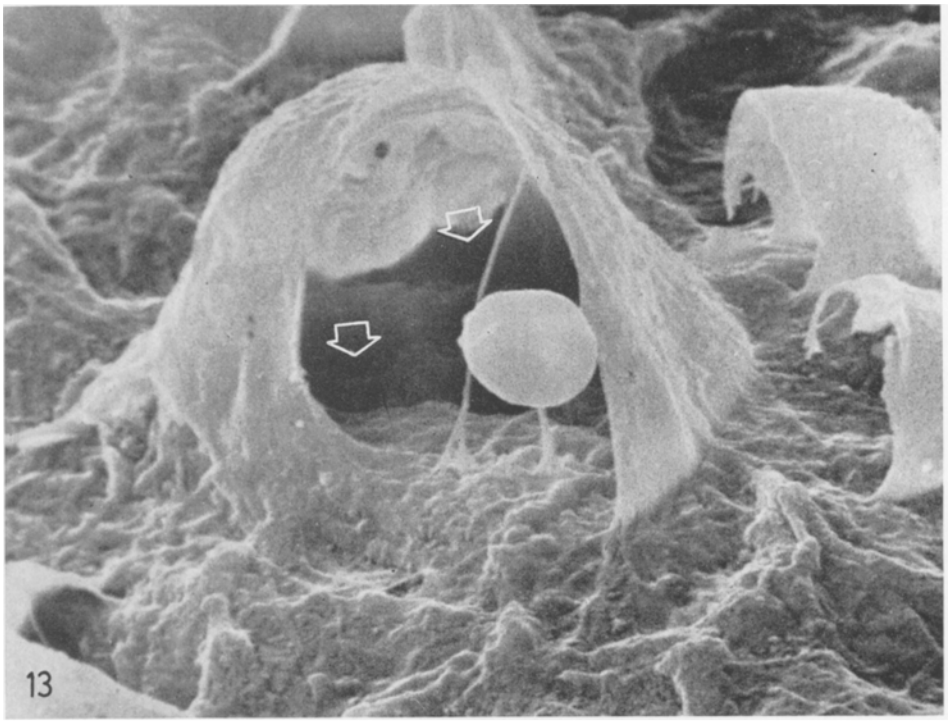
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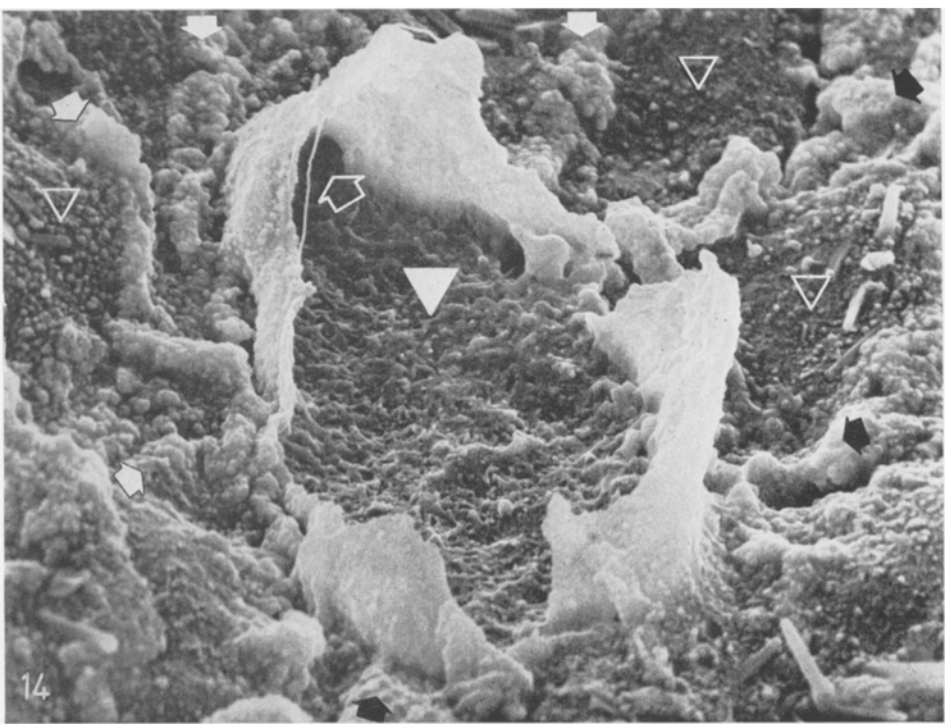
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served by S.E.M. was increased. The endothelial changes observed were almost similar to those described above. In the animals fed a cholesterol-containing diet for 4 weeks funnel-shaped mono- or multi-cellular changes were observed covering relatively small areas (Fig. 15).

c) Cholesterol-Containing Diet for 2–3 Weeks

In the aortas of animals fed a cholesterol-containing diet for 2 weeks hardly any lesions were observed in the S.E.M. In a few places single and multi-cellular endothelial changes were observed (Fig. 16). In the areas observed, the endothelial lining had a normal aspect, but at some places it bulged slightly into the lumen.

II. Transmission Electron Microscopic (T.E.M.) Observations

Descriptions of transmission electron microscopic appearances of relatively large endothelial lesions in rabbit aortas following administration of a cholesterol containing diet for 6–7 weeks are well known and will not be further described (Florentin *et al.*, 1968; Imai *et al.*, 1966; Marshall *et al.*, 1966).

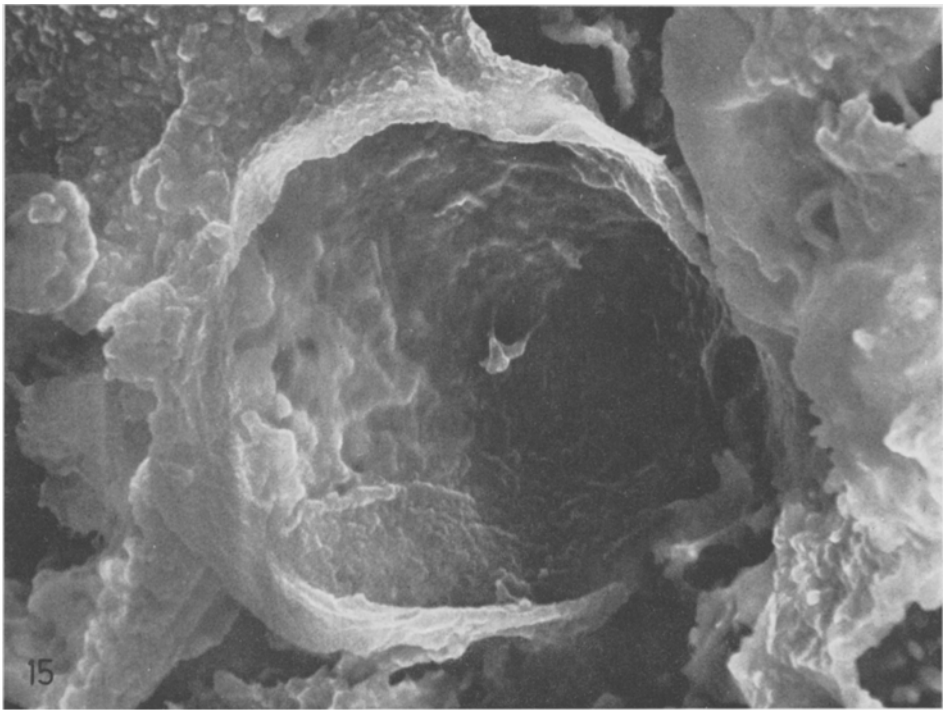
In addition to the large lesions much smaller lesions were observed characterized by the presence of round or oval, membrane-bound, lipid globules, which were present in the extra-cellular sub-endothelial space (not illustrated). The presence of such membrane-bound lipid globules in the extra-cellular spaces of the intima near the fenestrae in the internal elastic lamellae led us to suggest that these membrane-bound, lipid globules were the first, extra-cellular lipid depositions in these aortas (De Bruijn and Schornagel, 1969). The endothelial cells covering the lesions were more or less normal but bulged into the lumen. Similar globular lipid deposits were observed in the sub-endothelial space of rabbits fed a cholesterol diet for 4 weeks. An example of such an area is given in Fig. 18. In some places the presence of membrane-bound, lipid globules were observed which were apparently in a large vacuole between endothelial cells over this area (Fig. 17, arrow).

In the aortas of animals fed a cholesterol diet for 2–3 weeks intimal lesions were seldom found. Sub-endothelial lipid deposits of this type are, however, almost beyond the resolving power of the light-microscope and as consequence hard to find. Fig. 19 illustrates such an area of lipid globules in the sub-endothelial space. The lipid globules in this area were interspersed in between the normal constituents of the sub-endothelial space without apparently disturbing the endothelial lining. In most cases the endothelial lining over these rather minimal lesions was normal and did not bulge into the lumen. A few endothelial cells were observed with finger-like processes pointing into the lumen over areas where lipid globules were present in the sub-endothelial space.

Figs. 15 and 16. Endothelial changes in aortas with shorter cholesterol regimes

Fig. 15. Deep mono-cellular crater in a slightly elevated area of a rabbit aorta following 4-weeks cholesterol administration. The inside of the crater surface differs from the outside. Lipid material is not observed at the inside. (4 w. Cholesterol, Garbarsch, M × 3750)

Fig. 16. Mono-cellular and small multi-cellular change near to each other in a rabbit aorta following 2 weeks of cholesterol administration. (2 w. Cholesterol, Sinapius, M × 7500)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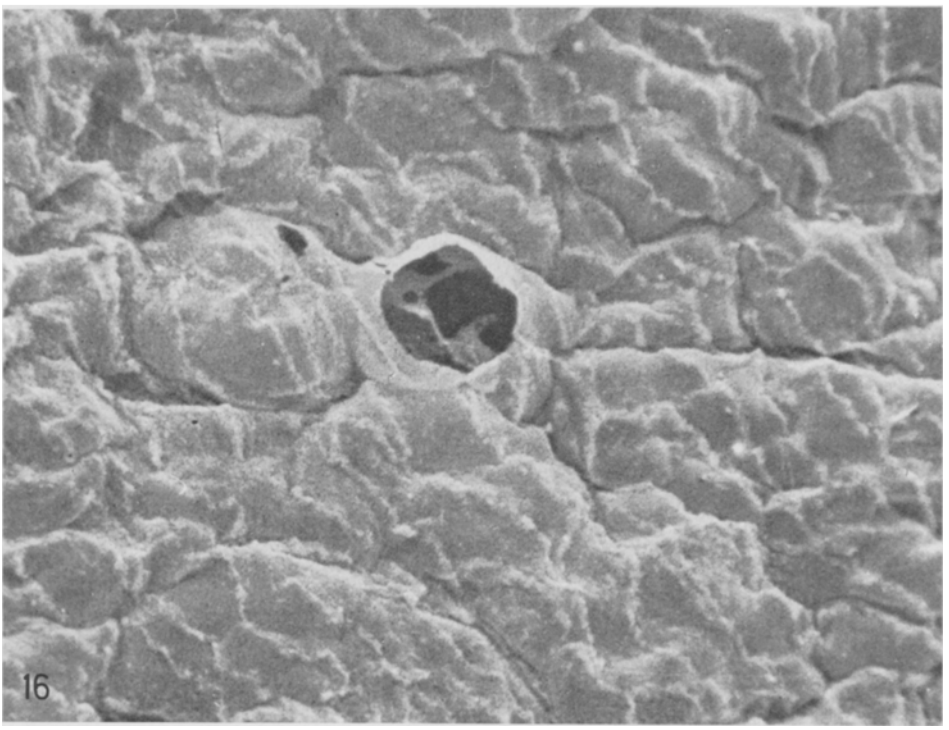
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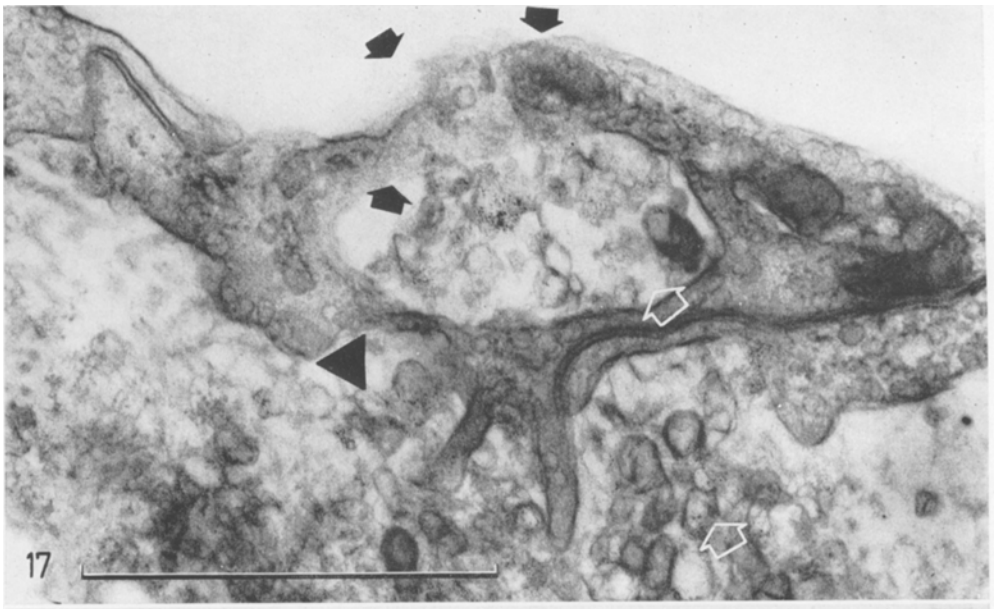
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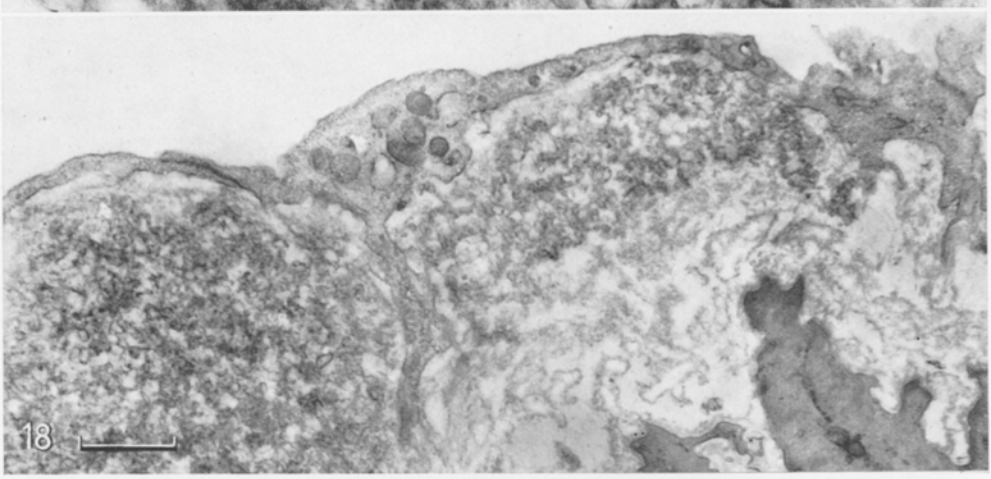
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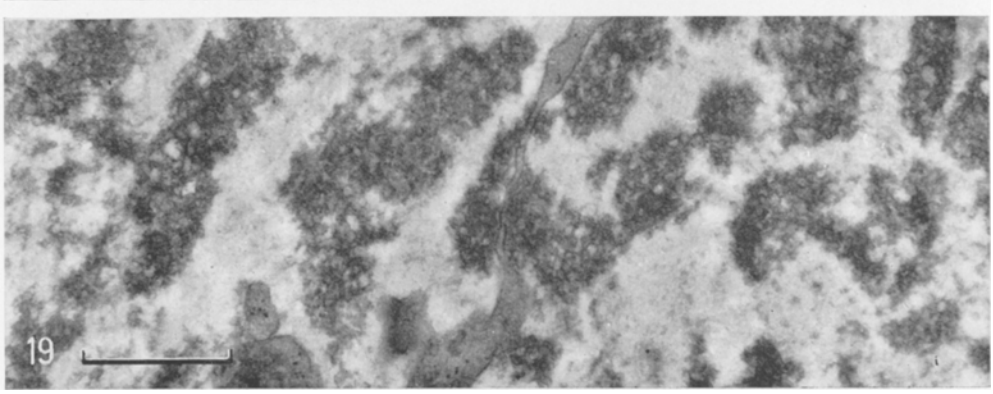
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Discussion

1. Observation of the endothelial cells in an "en face" view was always associated in the past with the study of endothelial cell changes induced by experimental or natural atheromatosis (Florey *et al.*, 1959; Poole *et al.*, 1958; Gottlob and Zinner, 1959) in order to investigate the role of the endothelial cells in this process. Acceptance that particulate serum lipids are deposited in the sub-endothelial zone of vessel walls, raises the question whether the lipid particles pass through or between the endothelial barrier (Gofmann and Young, 1963; Adams, 1967). T.E.M. studies have not been of great help in answering the question. In most cases the vascular lesions induced by prolonged cholesterol or lipid administration are found to be covered by endothelial cells. Although small areas have been observed without endothelial cover, usually the endothelial barrier has been found to be intact.

As the amount of lipid material found in the cytoplasm of the endothelial cells in such conditions is relatively small, it is assumed that it passed through the endothelial barrier between the cells. The question has been discussed by (Cotran, 1965; Florey, 1967; Haust, 1971; Watts, 1971). Although Adams (1973) has recently emphasized that damage of the endothelial barrier by a variety of causes (De Bruijn and Schornagel, 1966; Jellinek, 1973; Denes, 1973) increases its permeability, the question remains how individual endothelial cells react to damaging stimuli. It was hoped that S.E.M. studies of the endothelium might provide an answer to the problem of how the endothelial cell reacted to a barrier damaging agent, being present in rabbit aortas following several weeks of dietary cholesterol administration.

Our present study confirmed that the majority of gross atheromatous lesions were covered by an endothelial cell layer. But close observations revealed that delicate mono-cellular endothelial changes were present both on the surface of large intimal lesions and in endothelium covering normal or barely elevated intima. Mono-cellular changes were occasionally observed in normal aortas. Our observations suggest that the mono-cellular change represents a single cell reaction to a very locally applied stimulus. The frequent presence of mono-cellular changes over

Figs. 17–19. Transmission electron micrographs of similarly treated animals

Fig. 17. Sub-endothelial space with a deposit of small, membrane-bound, lipid globules. Note the vacuolar structure in the endothelial layer where similar membrane-bound lipid globules are present (⊲⊳). (▼) Note the thin piece of endothelial cell at the site of the sub-endothelial space that separates the vacuole from that space. Arrows point to the thin part of endothelial cell at the lumen site of that vacuole where the vague shadow over the sharp cell membrane indicates the tortuous shape of that endothelial cell part in the third dimension. (4 w. Cholesterol, M × 44600. Bar = 1 μ)

Fig. 18. Sub-endothelial deposit of small membrane-bound lipid globules. Note the typical horse-shoe shape of the endothelial cells bulging into the lumen due to their covering the deposits of lipid-particles (4 w. Cholesterol, M × 12900. Bar = 1 μ)

Fig. 19. Sub-endothelial deposits of small membrane-bound lipid globules. In this phase, clusters of lipid globules are still "immersed" into the normal extra-cellular components of the sub-endothelial space. (2 w. Cholesterol, M × 19800. Bar = 1 μ)

atheromatous lesions at sites facing the bloodstream rather than on down stream sites, suggests that the damaging agent is to be found in the bloodstream.

The presence of funnel or dome-shaped endothelial changes (Figs. 11, 13 and 14) in normally flat or slightly bulging endothelium suggested that mono-cellular changes not only contributed to the sub-endothelial storage of lipid material in large lesions but might even initiate the process in normal areas. The appearance of mono-cellular changes in aortas following 2–3 weeks cholesterol administration suggested that the damage to the endothelial cells resulted qualitatively in similar endothelial changes to those that followed prolonged cholesterol feeding, though the quantitative differences occurred.

The infrequent occurrence of mono-cellular endothelial changes, combined with the difficulties which arise in three dimensional reconstructions from transmission electron micrographs, may explain why the endothelial changes observed by the S.E.M. studies have not been described in T.E.M. studies.

After scrutinizing our files of T.E.M. photo-micrographs, two or three convincing pictures were found that could be interpreted as cross sections of such funnel-shaped endothelial changes with some particulate lipid material inside (Fig. 17). Moreover, when in such cases the thin ring-shaped endothelial cell layer opens on the intimal side (Fig. 17 ▼) the appearance of a cross sectioned funnel is lost. The horse-shoe-like appearance of the endothelial cells covering the deposits of globular lipids in the cross sectioned sub-endothelial spaces suggest the presence of such opened endothelial cell funnels. However, as yet, longitudinally sectioned aortic lesions have not produced the desired longitudinally sectioned funnel appearance.

Although some photomicrographs (Figs. 2, 7, 8, 9, and 13) suggest that the action of single endothelial cells resulted in funnel- or dome-shaped surface changes and lipids being channelled through the endothelial barrier, other photomicrographs (Fig. 14 and 16) indicated that small numbers of cells combined to form a structure which protruded into the lumen. In one case the surface appearance inside the endothelial cell fences (Fig. 14 arrow) was observed to be different from that on the outside. As lipid particles were not detected in that area the importance of such a structure in the process of lipid transport through the endothelial barrier could not be ascertained. It is impossible to exclude the possibility that some of the described dome-shaped structures are the end-products of the combined change in a small number of cells, especially when multiple endothelial cell changes were present in close proximity to each other (Fig. 4 and 11) or when large lipid particles passed through the barrier (Fig. 12).

From the extensive fields of dome-shaped cell-changes (Fig. 11) it appeared to us that the end-stage might be a structure resembling a completely closed igloo. As several of the domes exhibited small holes whereas other had none, it seemed that the open structures were a time related development.

The observed multicellular endothelial crater-like changes were already well recognized from earlier T.E.M. studies. The presence of large lipid containing macrophages inside the areas without endothelial cells had been observed previously in the T.E.M., in this study we have observed surprisingly large areas without endothelial covering (Fig. 5).

Perfusion damage as a cause for the changes cannot be completely dismissed as the endothelial covering of such lesions might be more sensitive to applied

mechanical forces than normal endothelium. However, the cells at the edges of large craters were round and several cell proliferations were observed suggesting a normal process of repair. The inside of these large craters is similar in structure to the inside of small craters (Fig. 14). However, fibrin threads were more frequently present inside large craters, whereas lipid particles were not detected. The contribution of these craters to the actual transport of lipid through the endothelial barrier cannot be ascertained.

It is uncertain whether the stimulus inducing these endothelial changes is present in the (altered) circulating fluid or is located in the vascular wall itself. Tosi and Weber (1973) recently presented evidence for the reduction of a cellcoat substance detectable at the endothelial cell surface covering atheromatous lesions. The relationship between the presence or absence of such a cellcoat and the methods of fixation employed was the subject of a critical study by Bernard *et al.* (1973) in viral infected fibrocytes. Damage to the cellcoat may be the stimulus that induces the endothelial cell reactions described above. The idea that endothelial cell response to a stimulus is a time-dependent process, as suggested by our observations, is still speculative.

In conclusion, several perforations of the endothelial cell barrier of different diameters which are most probably open for variable periods of time, were observed in rabbit aortas following the application of a damaging agent for two to seven weeks. Injury to a single endothelial cell or a small number of adjacent cells led to funnel- and dome-shaped cell protrusions through which lipid particles were channelled. As the material transported was assumed to be surrounded by the dome- or funnel-shaped endothelial cell protrusions, it probably neither entered the actual endothelial cytoplasm nor passed through existing inter-endothelial spaces.

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