

Electron Microscopy of the Spleen

II. Phagocytosis of Colloidal Carbon

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COLLOIDAL PARTICLES, such as carbon, have been used for many years to study the physiology and phagocytic capacities of the reticuloendothelial system.^{1,2} The method by which the particulate matter is cleared from the blood has been deemed important because of its analogy to septicemia and to the clearance of antigens in immunologic reactions.³ Although there have been a considerable number of studies on the physiology of reticuloendothelial phagocytosis, there have been relatively few ultrastructural studies and most of these have concentrated on the liver and not on the spleen.⁴⁻¹⁰ The only electron microscopic studies emphasizing splenic phagocytosis have been concerned with reticuloendothelial blockade¹¹ or anatomical investigations.^{12,13}

Tracers have also been used to localize and follow the movement of antigen within the spleen at different periods following injection. The distribution of I¹²⁵ labelled *Salmonella flagellar* antigen has been compared with that of carbon particles.^{14,15} Both *Salmonella* antigen and carbon accumulate initially in the red pulp and marginal zone but differ in their final localization. This movement of tracer containing macrophages has not been verified with the electron microscope in the spleen. Nor have there been any studies to determine if it is only the macrophages that migrate, or whether, for example, the splenic endothelial cells also participate in any migration and phagocytosis. It is also curious that there have been more studies of phagocytosis by endothelial cells outside the reticuloendothelial system.¹⁶⁻¹⁹

Materials and Methods

Intravenous injections of a nontoxic suspension of colloidal carbon (C11-1431a, Guenther Wagner, Pelikan-Werke, Hanover, Ger.) were given to 10 albino rabbits in a dose of 1 ml/kg (10 mg carbon per 100 gm body weight). Spleens were

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removed after 20 sec, 30 sec, 1 min, 1½ min, 3 min, 6 min, 1 hr, 12 hr, 24 hr, or 1 week following the injection, and placed immediately in cold buffered 1% osmium tetroxide, and cut into 1 mm slices. To achieve splenectomy in the shortest possible times after carbon injection (20 and 30 sec) it was necessary to administer a sleeping dose of intravenous sodium pentobarbital in one ear vein and then inject the carbon through the opposite ear vein. A killing dose of sodium pentobarbital immediately followed into the first ear vein. Cardiac arrest occurred 5 sec after the carbon injection in the fastest experiment and it took another 15 sec to complete the laparotomy and immersion of the spleen in cold buffered 1% osmium tetroxide. In all cases the kidney was also obtained for examination and processed for electron microscopy. Sections from kidney, liver, lung, and spleen were also fixed, embedded, and stained for light microscopy.

Following fixation of the tissues for electron microscopy in cold buffered 1% osmium tetroxide for 1 hr, the tissue fragments were dehydrated in graded alcohols and propylene oxide according to the method of Luft,²⁰ and were embedded in a mixture of Epon-Araldite as described by Givan, Turnbull, and Jézéquel.²¹ Sections were cut with glass or diamond knives on a Porter-Blum MT1, LKB or Reichert OmU2 ultramicrotome. Thick sections (0.5 to 1.0 μ) were stained with toluidine blue. Sections for electron microscopy were stained either with lead hydroxide²² or lead citrate.²³ These sections were examined and photographed in an RCA EMU-3F or Philips EM-300 electron microscope.

Results

Twenty and 30 sec following intravenous injection, the carbon localizes predominantly in tiny clumps in the sinuses of the marginal zone and red pulp. At this stage some carbon is already being phagocytosed by macrophages arising from the Billroth cords. These macrophages extend fingerlike projections or pseudopods through the fenestrations of the sinus basement membrane and between the endothelial cells of the sinus into the lumen (Fig 1-3). At this time, carbon is observed to lie in cytoplasmic invaginations in these macrophagic prolongations and some particles are even present within the cells in intracytoplasmic vacuoles (Fig 1-3). Small blebs may form at the tip of the macrophage during this phase of very active phagocytosis. At a later stage, the carbon in the macrophages comes to lie predominantly in single membrane bound vacuoles, though occasionally a vacuole has a double membrane (Fig 4). Many of the vacuoles are tubular in appearance creating the impression that the carbon may be in the dilated smooth endoplasmic reticulum.

Platelets in aggregation without fibrin formation are often found in close proximity to the pseudopods of macrophages in the sinus lumens. Carbon particles are found around the periphery of the platelets, and some particles are phagocytosed by the platelets and appear to lie within their tubular system (Fig 5). The platelets in the aggregate may have a normal disposition but generally concentrate their granules

towards the center of the cell and form multiple blebs. Carbon particles may also be seen within these blebs.

At 20 and 30 sec some carbon particles are also present within the cords. These particles undergo phagocytosis in the same way as in the lumens of the sinuses, through the formation of multiple surface membranous invaginations in macrophages. Occasionally carbon particles lie free in large masses or clumps within the sinuses, even adjacent to the capsule.

At 1 min carbon is still found free in the lumens of the sinuses but a greater quantity is in phagocytic vacuoles in the intraluminal extensions of the macrophages. Some of these macrophages even arise from between the capsule and subcapsular sinuses (Fig 6). At this time some of the macrophage projections containing ingested carbon are beginning to retract back into the Billroth cords and are frequently accompanied by platelets (Fig 7). Platelets in aggregation and still ingesting carbon particles are found even at 1 min 30 sec following injection.

Free or floating macrophages are frequently seen in the sinuses at 3 min (Fig 8). These macrophages contain injected carbon particles as well as degraded erythrocytes and/or leukocytes and may reach enormous size. Free macrophages are found at later times, but appear more numerous at the 3 min interval. At 3 min, electron microscopy of the kidney reveals free carbon in glomeruli. Some carbon is being phagocytosed by platelets in the glomeruli. Carbon is also found in the capillaries of the renal interstitium and in neutrophil phagosomes. Phagocytosis of carbon by neutrophils is only rarely even found in the spleen. By 3 min, the phagocytosis of carbon by platelets in the spleen is considerably diminished, although there still are multiple platelet aggregates both in sinuses and cords, without carbon and without fibrin formation. Phagocytosis by macrophages of carbon containing platelets was not observed.

At 6 min following the carbon injection, the particles are still concentrated in the marginal zone and red pulp, and may still lie freely in huge clumps or masses, both in the sinuses and cords. Phagocytosis is continuing in the pseudopods of macrophages extending from the cords, and for the first time a few intracytoplasmic carbon particles may be found in a sinus endothelial cell (Fig 9). Carbon particles are also found in macrophages within the cords.

At 1 hr there is an increase in the amount of carbon in individual macrophages, both in the cell bodies and in their pseudopodal extensions. This is most marked in the cord macrophages, especially

those immediately beneath the basement membrane. Here, the vacuoles become more globular in shape, and coalesce especially at the base of the cell away from the sinus. Again, there is a minimal amount of carbon in endothelial cells of the sinuses.

By 12 hr there is a tremendous increase in the quantity of carbon in the macrophages of the Billroth cords. The carbon may be in individual phagocytic vacuoles or in large coalesced vacuoles (Fig 10). Some carbon containing vacuoles achieve considerable size. In these vacuoles, although carbon is seen throughout, the majority is concentrated at the periphery and the central areas are relatively clear and empty. Within these clear areas are small fragments of membranous appearing material which may be the remnants of the smaller vacuoles. Endothelial phagocytosis of carbon particles, although small, is at its maximum at this time (Fig 10, inset). The carbon containing vacuoles in the endothelium are relatively close to the luminal surfaces and in close proximity to the pinocytotic vacuoles. No other cytoplasmic changes are noted in these cells. Pseudopodic extensions of macrophages into the lumen are not seen at this stage.

Carbon containing macrophages are found in the periphery of the white pulp by 24 hr. The carbon may be arranged in large multiple coalescent vacuoles as was seen in the red pulp at 12 hr. More commonly, however, there is only one large vacuole in the macrophage, with a clear center and carbon concentrated at the periphery (Fig 11). These macrophages containing a single vacuole are more frequent in the peripheral white pulp than in the red. The surrounding lymphocytes, reticulum cells, and fibroblasts are unremarkable and there are no noticeable reactive changes.

The final stage examined was 1 week. At this stage there is an even more marked increase in the amount of carbon in the spleen, and it is particularly concentrated in the marginal zones and red pulp. The carbon in the white pulp is peripheral and very slight in quantity in comparison to the red pulp. There appears to be no more in the white pulp at 1 week than was found at 24 hr. Within the marginal zones and red pulp no free carbon is present. All carbon particles lie densely packed in vacuoles of varying sizes within the macrophages of the Billroth cords and marginal zones (Fig 12). In these macrophages almost no carbon-free cytoplasm can be discerned. The cells appear stuffed with the black particles. A curious finding is the interdigitation of the cell membranes of adjacent carbon containing macrophages.

The liver, kidney, and lung were examined with the light microscope at all phases of these injections. It was found that as there was a marked

increase in the amount of carbon in the spleen by 1 week, there was a simultaneous marked loss of carbon from the other organs.

Discussion

Electron microscopy of the spleen at different time intervals following the intravenous injection of colloidal carbon has yielded three principal findings: (1) phagocytosis in the spleen is extremely rapid; (2) splenic phagocytosis of carbon occurs almost exclusively in the macrophages of the Billroth cords and only very very minimally in endothelial cells; and (3) platelets may play an important role in the initial sequestration of carbon in the spleen.

Twenty and 30 sec following the injection, some carbon particles had already been phagocytosed by macrophages in the Billroth cords or by platelets in the sinuses. A more common finding was carbon at the cell membrane of the phagocyte or in clefts or invaginations of its cell surface. In transilluminated spleens, India ink has been seen inside pulp phagocytes and on strands of reticulum as early as 14 sec after the injection had begun.²⁴ The only demonstration of early phagocytosis with electron microscope is that of Parks and Chiquoine,²⁵ who demonstrated phagocytosis of colloidal gold in the liver within 15 sec following injection.

Virtually all the carbon in the spleen was phagocytosed by macrophages from the Billroth cords and marginal zones and not by sinus-lining endothelial cells. The macrophages from the cord extend finger-like projections through the apertures in the sinus basement membrane and between the endothelial cells of the sinus in order to ingest carbon in the sinus lumen. These pseudopodic extensions into the sinuses were mistaken for endothelial cells in early studies by light microscopy.²⁶ Their presence also explains the failure to find acid phosphatase activity in the endothelial cells of the spleen.²⁷ The present study has also demonstrated that the macrophages which phagocytose carbon may contain phagocytotic debris from previous erythro-leukophagocytosis.²⁸

The distribution of carbon in the macrophages of the red pulp, marginal zone, and the periphery of the white pulp conforms to transillumination and silver impregnation studies,^{24,29} but disagrees with those of Nossal *et al.*¹⁵ These investigators found carbon distributed throughout the entire white pulp of the rat in a light microscopic study. The explanation for this difference is not clear, but it may be due to tangential sections or species differences. This present study does however answer certain questions posed by Nossal and his co-workers. The phagocytic cells are macrophages, and these cells may easily migrate

from the red pulp to the peripheral zone of the white pulp and may become stellate in shape like a reticulum cell.

It should be noted that some carbon was found in endothelial cells in this study as early as 6 min following injection, and that the endothelial uptake became maximal at 12 hr. There was no increase in the amount in the endothelial cells thereafter, though the quantity in the macrophages continued to increase. It would appear that the endothelial cells of the spleen are no different, and have no greater capacity for phagocytosis, than any other endothelial cell in any other organ. Cotran¹⁸ has demonstrated endothelial phagocytosis of carbon in the capillaries of the endocardium and myocardium, but only after an overload of this material. In another study endothelial phagocytosis was found in the late phases of histamine-induced inflammation.¹⁹ One investigation showed one picture of endothelial phagocytosis in the spleen using saccharated iron oxide as a tracer but this occurred at 24 hr and after eight injections of the material.⁶ Similar results were obtained by Florey.⁹ In the present study, with time, the amount of carbon in the spleen increased, while the amount in the liver and lung decreased. Similar findings have been found using polystyrene latex particles.⁷ The shift of particles to the spleen from the liver and other organs must be due to re-entry of the particles into the circulation. This may reflect the superior capacity of the spleen for phagocytosis.² As splenic endothelium has no more phagocytic power than has endothelium in other organs, the use of the term "reticuloendothelial" to describe the endothelium of splenic sinuses is therefore a misnomer. Virtually all splenic phagocytosis is due to the macrophages, not the endothelium.

Finally, the presence of platelets and platelet aggregates around the carbon particles in the early phases of these injections provides a morphologic answer to the finding that the injection of carbon results in a decrease in circulating platelets.^{30,31} The platelet aggregates around carbon particles with phagocytosis of some of the carbon particles by the platelets in the spleen and in the kidney probably accounts for this decrease in platelets. Other investigators have previously shown in vitro that platelets are capable of phagocytosing such particles as latex, carbon, and antigen-antibody complexes.³²⁻³⁴ The present study demonstrates in vivo phagocytosis.^{32,35} The fate of these aggregated platelets and carbon containing platelets remains unknown. No increased phagocytosis of platelets containing carbon was found in the macrophages.

It has been shown that transfusion of platelet rich plasma results

in an increased rate of carbon clearance, and conversely administration of adenosine diphosphate reduces the clearance rate of carbon.³¹ From these findings and from the present study it appears that platelets are probably important in the transport of carbon to the R(E) system.

Summary

The phagocytosis of colloidal carbon by splenic macrophages takes place within 20 and 30 sec following intravenous injection. Carbon localizes initially in the sinuses of the marginal zones and red pulp and later within the Billroth cords. At 24 hr, the carbon is also found in the periphery of the white pulp but is never found diffusely through this area. Most of the carbon is phagocytosed by macrophages from the Billroth cords which extend pseudopods through the apertures in the basement membrane and between the sinus endothelial cells into the sinus lumen. These macrophage projections are usually in close proximity to platelets which aggregate around carbon particles and also participate in phagocytosis. The platelets may play an important role in transport of carbon to the macrophages. Endothelial cells of the splenic sinuses function like endothelial cells in any other organ. These cells phagocytose only a minimal amount of carbon and only after a functional overload. The spleen therefore is physiologically a reticuloendothelial (RE) organ, but its endothelium is E and not R.

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[Illustrations follow]

Legends For Figures

Fig 1. Rabbit spleen; marginal zone. Carbon injection (20 sec). Macrophage of marginal zone (MZ) is projecting pseudopod through gaps in basement membrane (bm) and between endothelial cells (E) into lumen of sinus (S). Carbon particles (arrows) are present at cell membrane and some particles (*large arrow*) are intracytoplasmic in a vacuole. Lymphocytes (Ly). Lead hydroxide stain. $\times 20,000$.

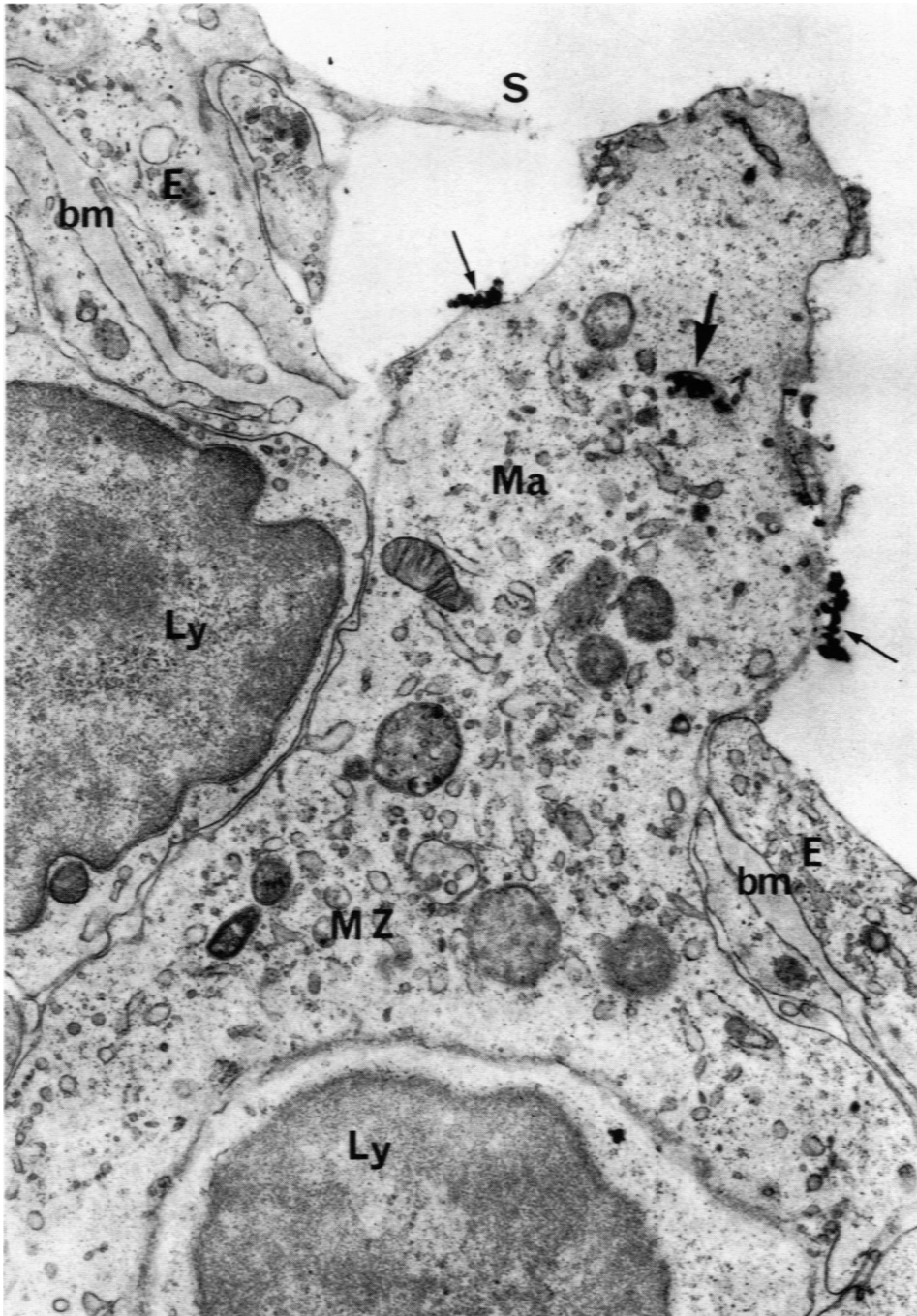


Fig 2. Rabbit spleen; red pulp. Carbon injection (20 sec). Macrophage (*Ma*) from Billroth cord (*BC*) is seen projecting pseudopod through pore in basement membrane (*bm*) and between endothelial cells (*E*) into sinus (*S*). Tip of this pseudopod is irregular and forms multiple small blebs (*bl*) and mazes of invaginations in which carbon from sinus is being actively phagocytosed (*large arrow*). Macrophage also contains multiple vacuoles (*V*) and myelinic figures (*arrow*) as a result of previous phagocytosis of erythrocytes and/or leukocytes. Lead hydroxide stain. $\times 27,500$.

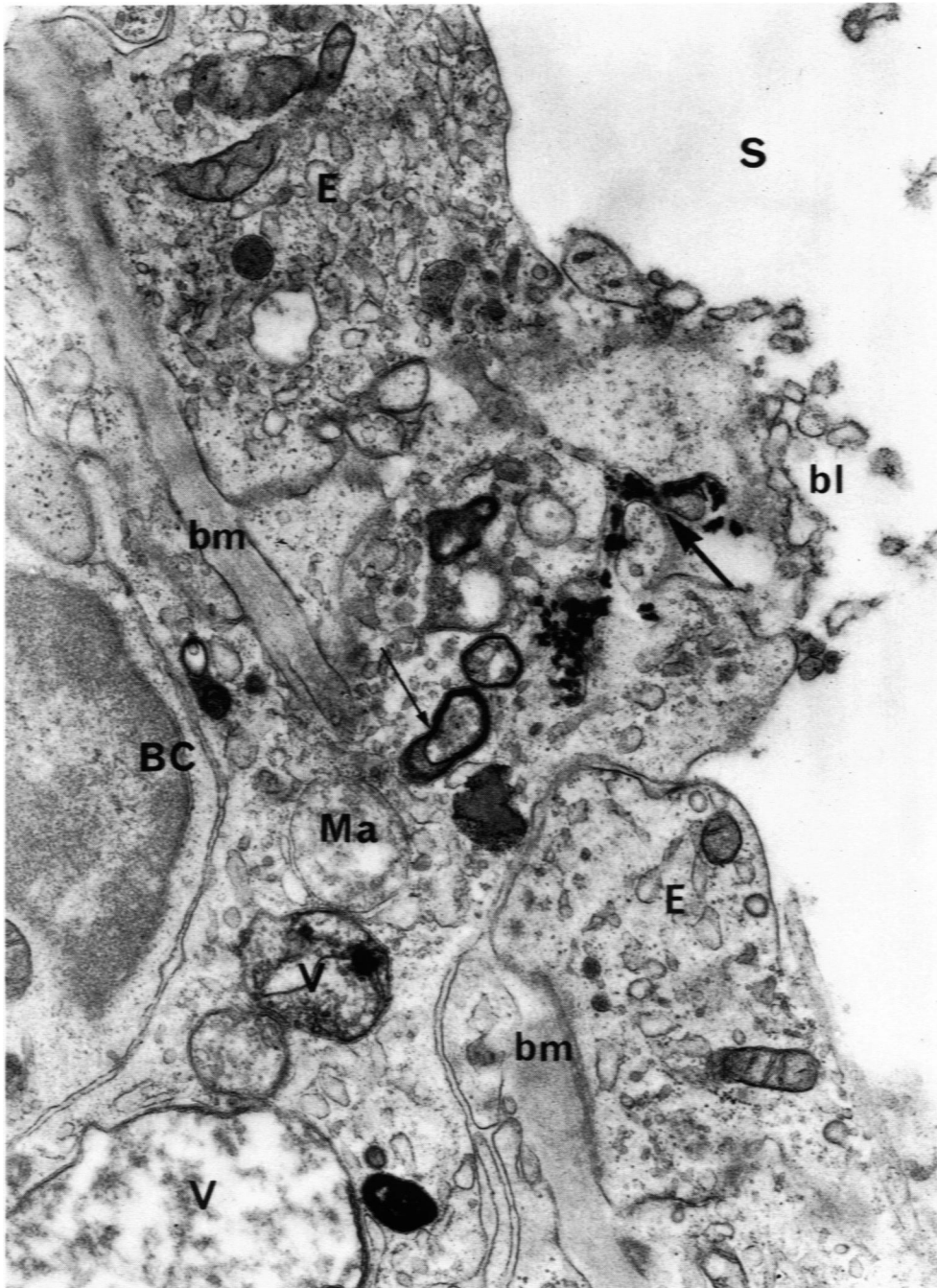
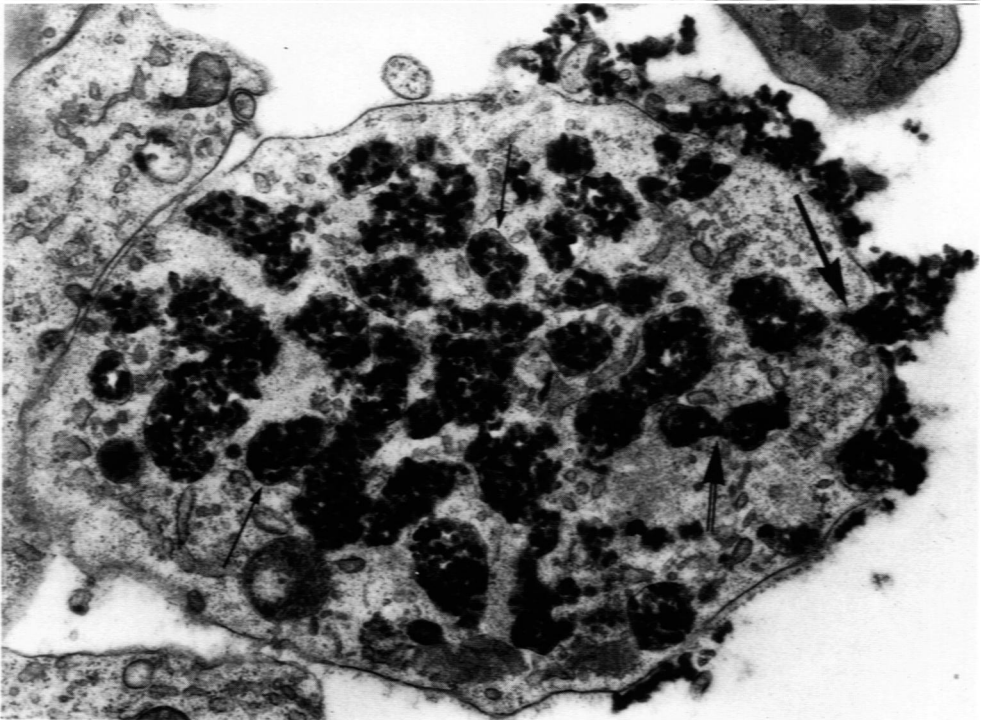
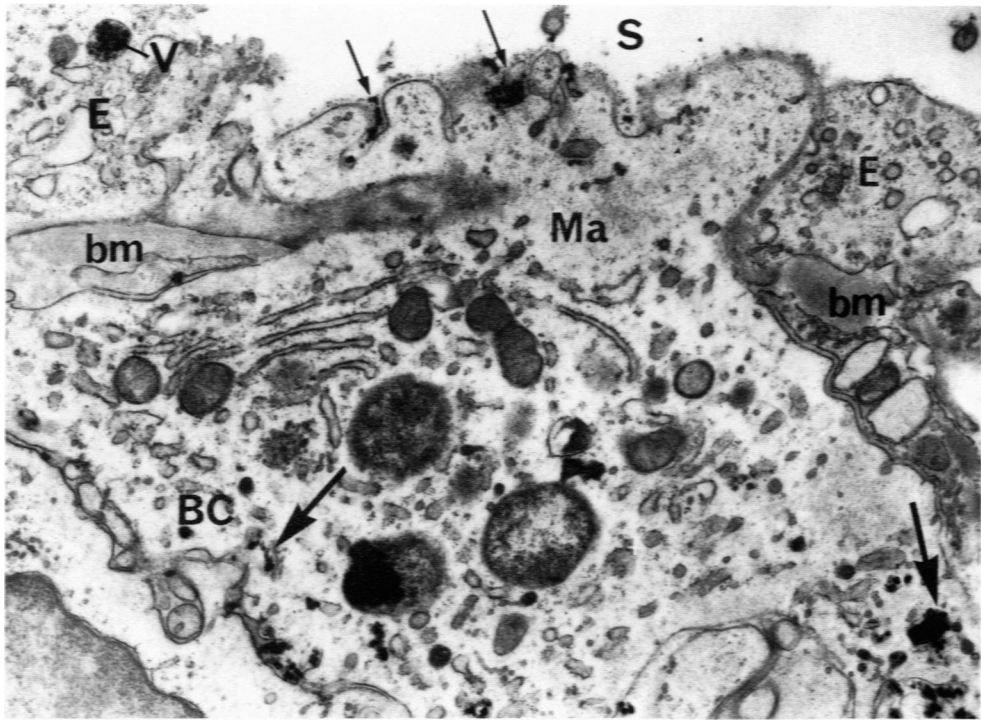


Fig 3. (*upper*) Rabbit spleen; red pulp. Carbon injection (30 sec). Cytoplasmic projection of macrophage (*Ma*) extends from Billroth cord (*BC*) through gap in basement membrane (*bm*) and between two endothelial cells (*E*) into sinus (*S*). At luminal surface of macrophage there are several small cytoplasmic invaginations within which are located tiny clumps of carbon (*arrows*). A few particles of carbon are already phagocytosed (*large arrows*). One endothelial cell contains fine dense granular inclusion in a vacuole (*V*). Lead citrate stain. $\times 15,000$.

Fig 4. (*lower*) Rabbit spleen; red pulp. Carbon injection (6 min). Detail of cytoplasmic projection (pseudopod) of macrophage in sinus demonstrates the heterogeneity of carbon containing vacuoles. Carbon lies in single membrane-bound vacuoles some of which are globular in shape (*arrows*) and others tubular in appearance (*double arrow*), creating impression that carbon may be in dilated smooth endoplasmic reticulum. Some carbon particles are free in sinus and against cell membrane which is invaginated in this location. Lead hydroxide stain. $\times 22,000$.



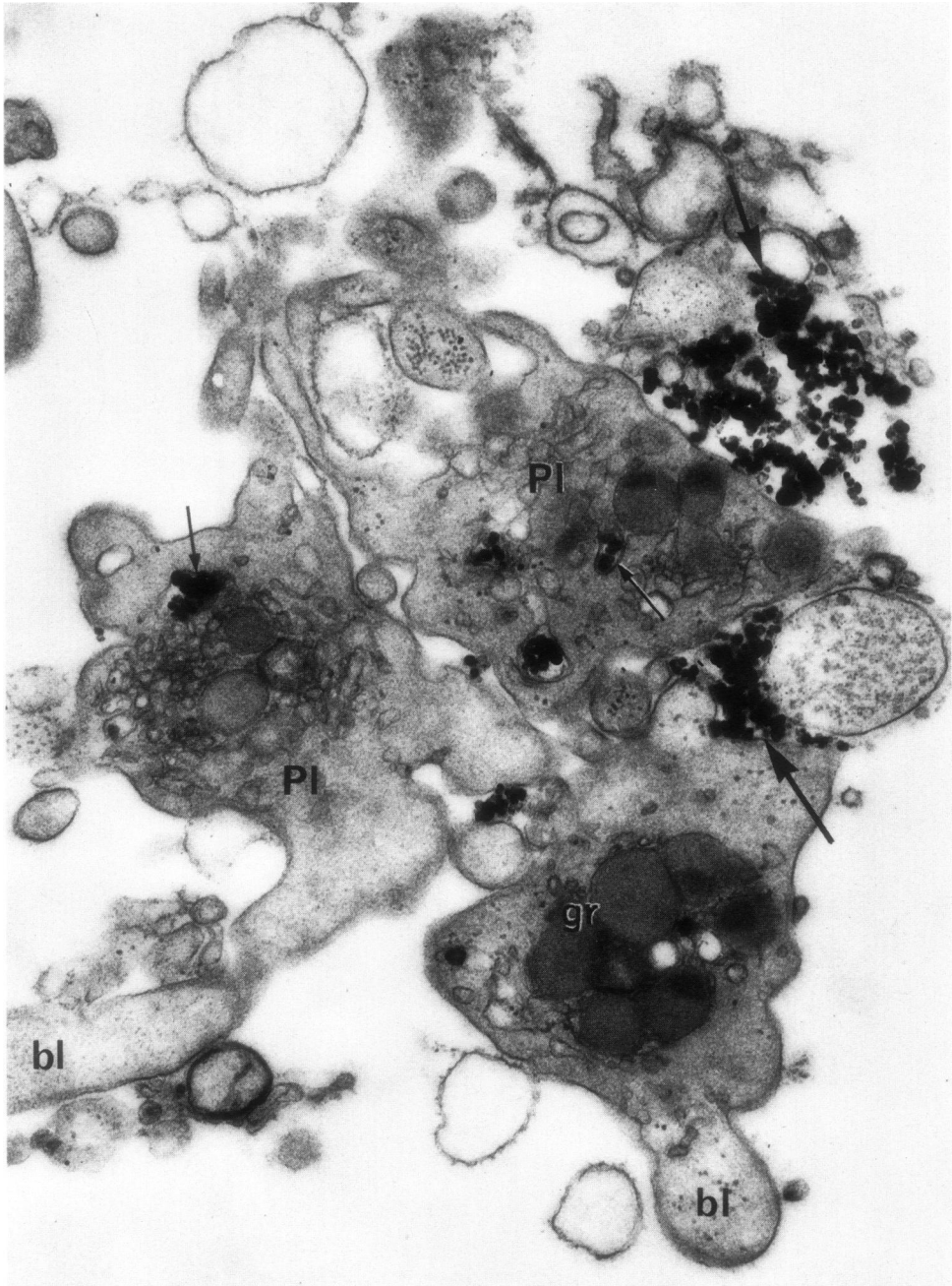


Fig 5. Rabbit spleen; red pulp. Carbon injection (1 min, 30 sec). In a sinus, platelets (Pl) are aggregating with carbon particles. Particles are present both outside platelets (large arrows) as well as inside (arrows). Injected carbon is located in platelet tubular system. Platelets in aggregation demonstrate formation of blebs (bl) and concentration of granules (gr). Lead hydroxide stain. $\times 32,000$.

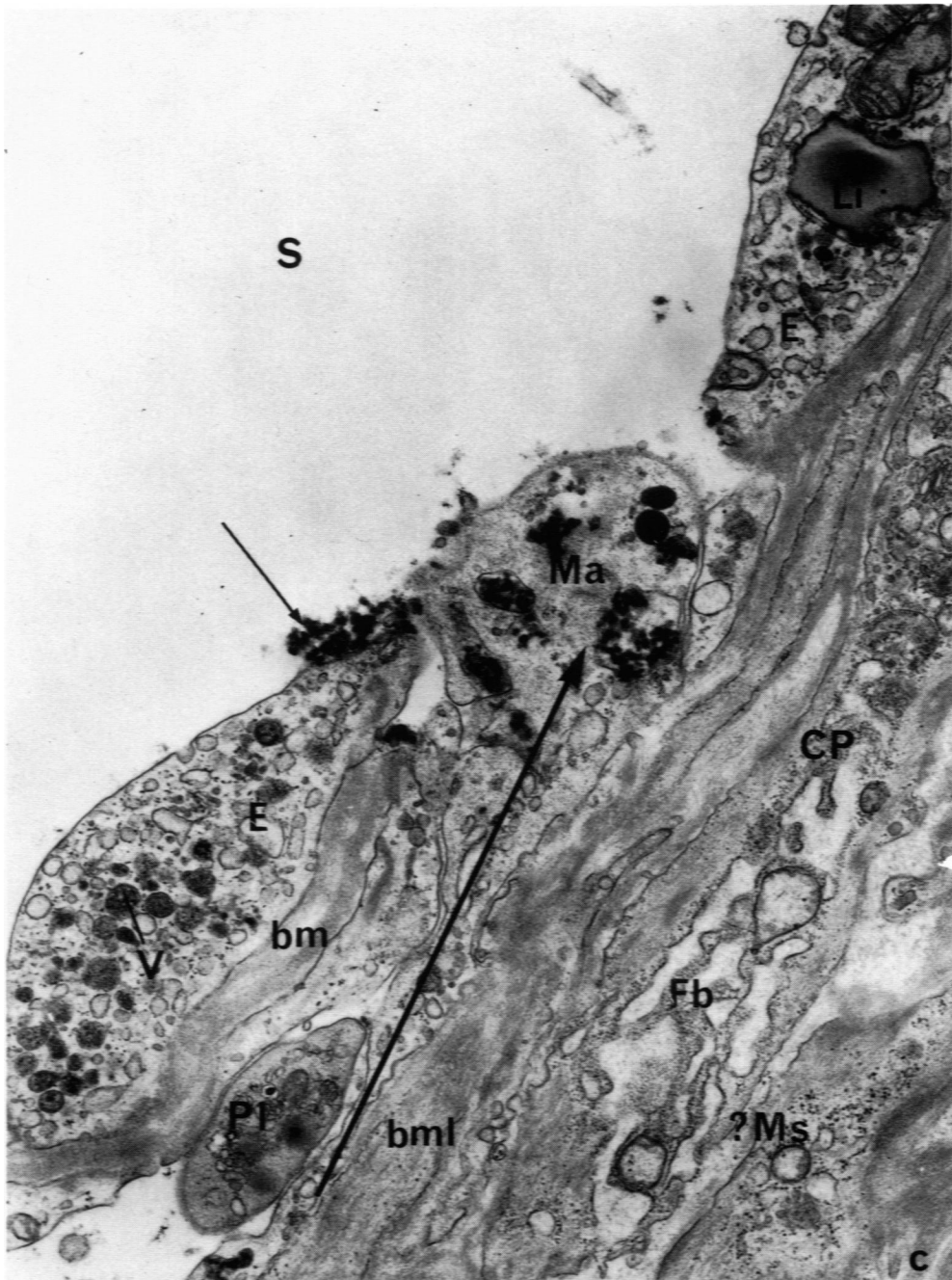


Fig 6. Rabbit spleen; capsule. Carbon injection (1 min). In subcapsular sinus (S), free carbon (arrow) is being phagocytosed by macrophage (Ma) which is extending (large arrow) from between basement membrane (bm) of sinus and between basement membranelike material (bml) of capsule (CP). Platelet (Pl) is also seen in this location. Endothelial cells (E) of sinus contain lipidic inclusions (Li) and granular material in small vacuoles (V), but no carbon. Capsule contains fibroblast (Fb), a smooth musclelike cell (?Ms) and collagen (c). Lead hydroxide stain. $\times 17,000$.

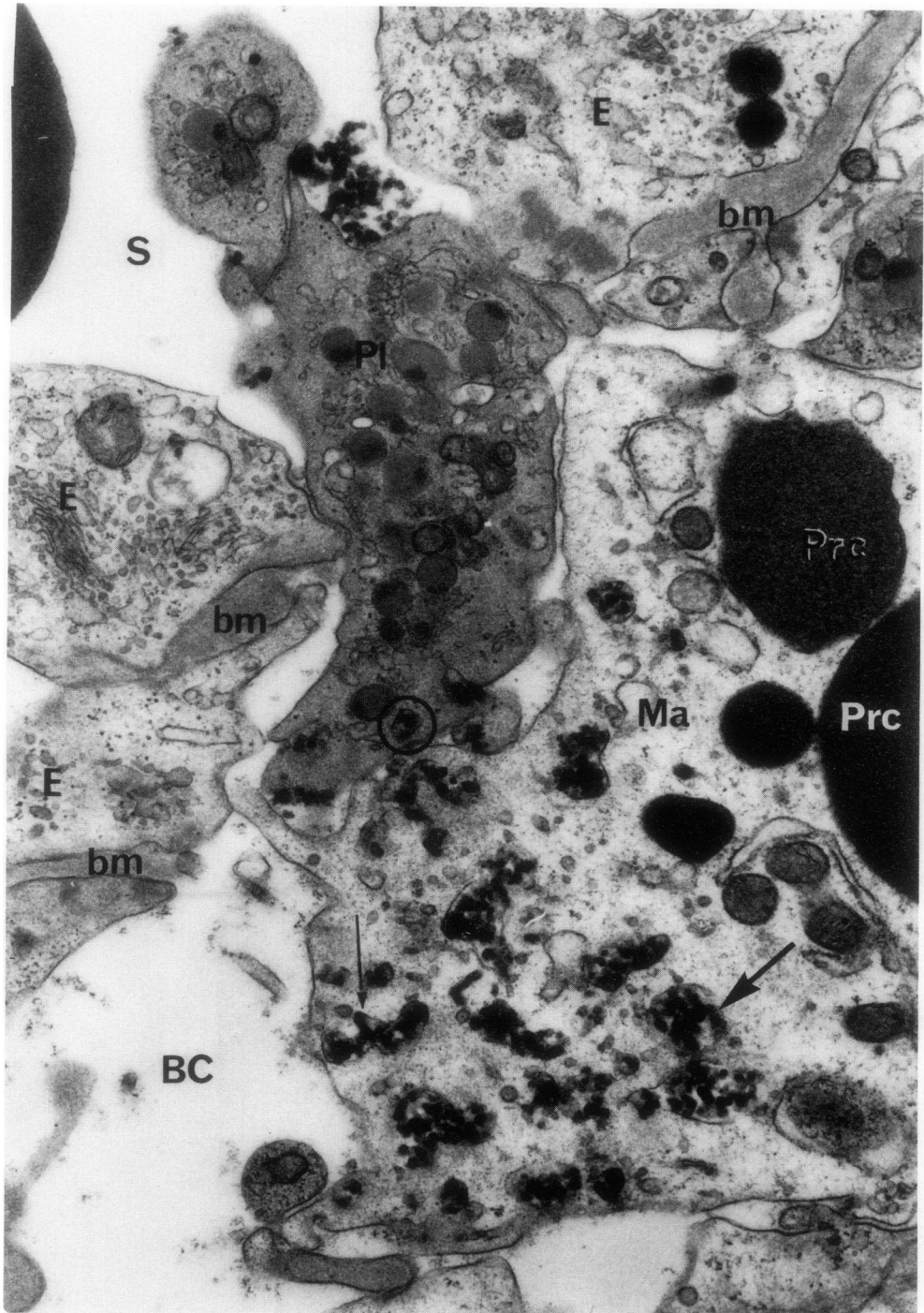


Fig 7. Rabbit spleen; red pulp. Carbon injection (1 min). Sinus is separated from Billroth cord (BC) by endothelial cells (E) and basement membrane (bm). Basement membrane contains large fenestrations. Lying between two endothelial cells in gap in basement membrane is a large platelet (PI). On luminal side of platelet, free carbon is present in sinus, while portion of platelet in Billroth cord contains ingested carbon (circle). Immediately adjacent to platelet in cord is a macrophage (Ma) which contains multiple carbon inclusions, some of which seem to be in smooth endoplasmic reticulum (arrow) while some are in large vacuoles (large arrow). Macrophage also contains several phagocytosed red blood cells (Prc). Billroth cord (BC) contains large channels filled with plasma. Lead citrate stain. $\times 27,000$.



Fig 8. Rabbit spleen; red pulp. Carbon injection (3 min). Free macrophage (*Ma*) in sinus contains phagocytosed carbon particles and phagosomes with old red cell debris (*Prc*). Carbon lies in tubular (*large arrow*) and vesicular (*arrows*) enclosures. Note the prominent Golgi zone (*G*). Lead hydroxide stain. $\times 16,000$.

Fig 9. Rabbit spleen; red pulp. Carbon injection (6 min). Carbon is being phagocytosed by portion of macrophage (*Ma*) lying in sinus (*S*). Cell is most likely part of macrophage lying immediately to the left (*Ma*₁), passing its cytoplasmic prolongation through pores of basement membrane (*bm*). Note paucity of organelles in these active macrophages as compared to endothelial cells (*E*), one of which contains a few intracytoplasmic carbon particles (*circle*). Carbon is also present in another macrophage (*Ma*₂) beneath basement membrane in Billroth cord. Lead hydroxide stain. × 15,000.

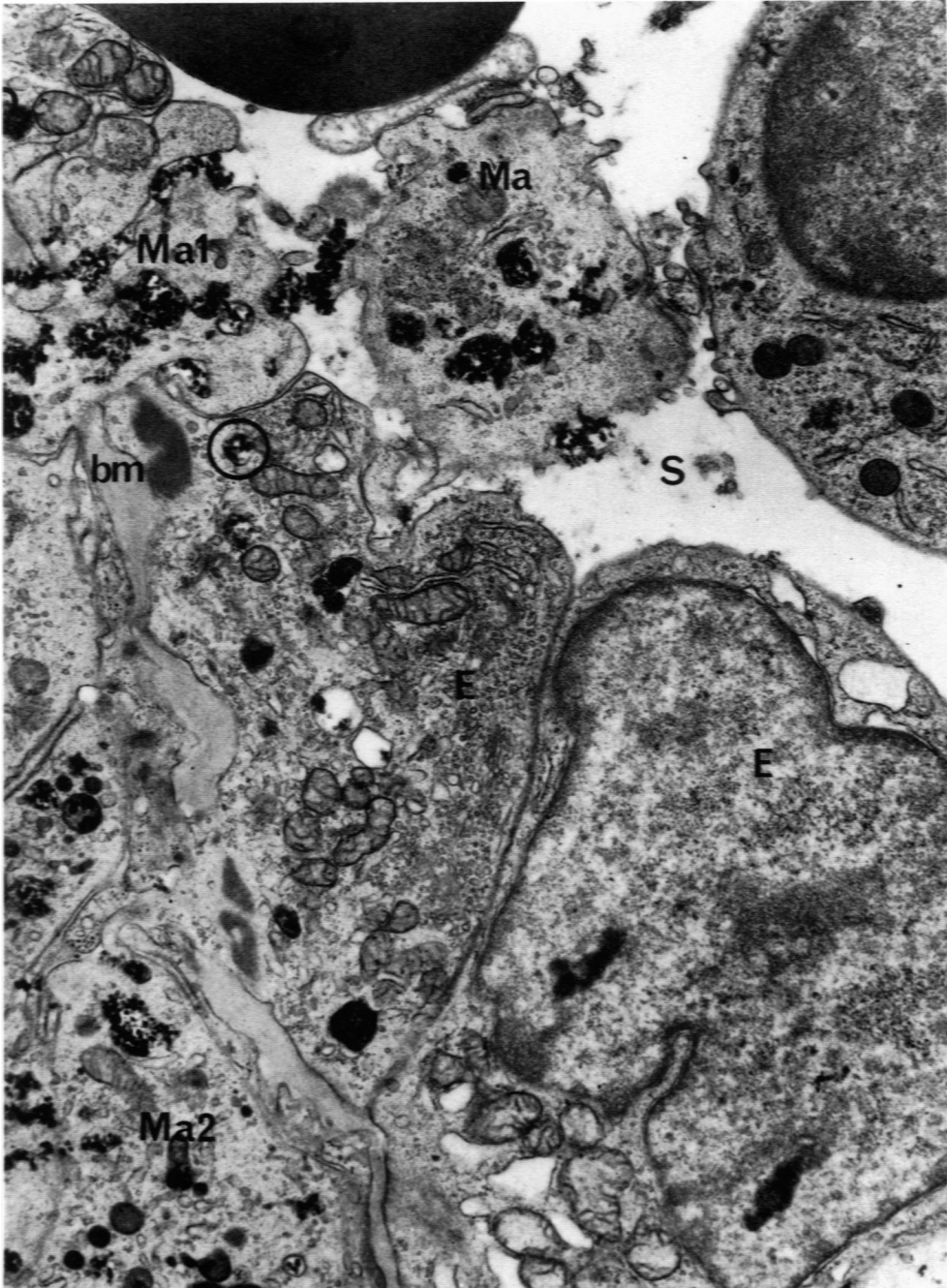


Fig 10. Rabbit spleen; red pulp. Carbon injection (12 hr). Macrophage (*Ma*) in Bill-
roth cord demonstrates tremendous increase in amount of carbon phagocytosed by
this time. Carbon lies in poorly defined vacuoles which are coalescing into larger
vacuoles. These vacuoles appear empty in an irregular fashion and within these clear
areas small membranes are present (*large arrow*) which may represent the remnants
of smaller vacuolar membranes. Within endothelial cells (*E*), there is an increase in
phagocytosed carbon (*arrow*). Sinus (*S*); basement membrane (*bm*); stellate retic-
ulum cell (*Rc₁*); reticulum cell (*Rc₂*). Lead hydroxide stain. $\times 15,500$.

Inset demonstrates detail of endothelial phagocytosis of carbon particles which
is maximal at this time. Carbon lies in vacuoles which are in close proximity to
pinocytotic vesicles (*arrow*). Cytoplasm of endothelial cell (*E*) shows no increase in
number of organelles. Some carbon is still free in sinus (*S*) and appears about to be
phagocytosed (*large arrow*). Basement membrane (*bm*). Lead hydroxide stain \times
30,000.

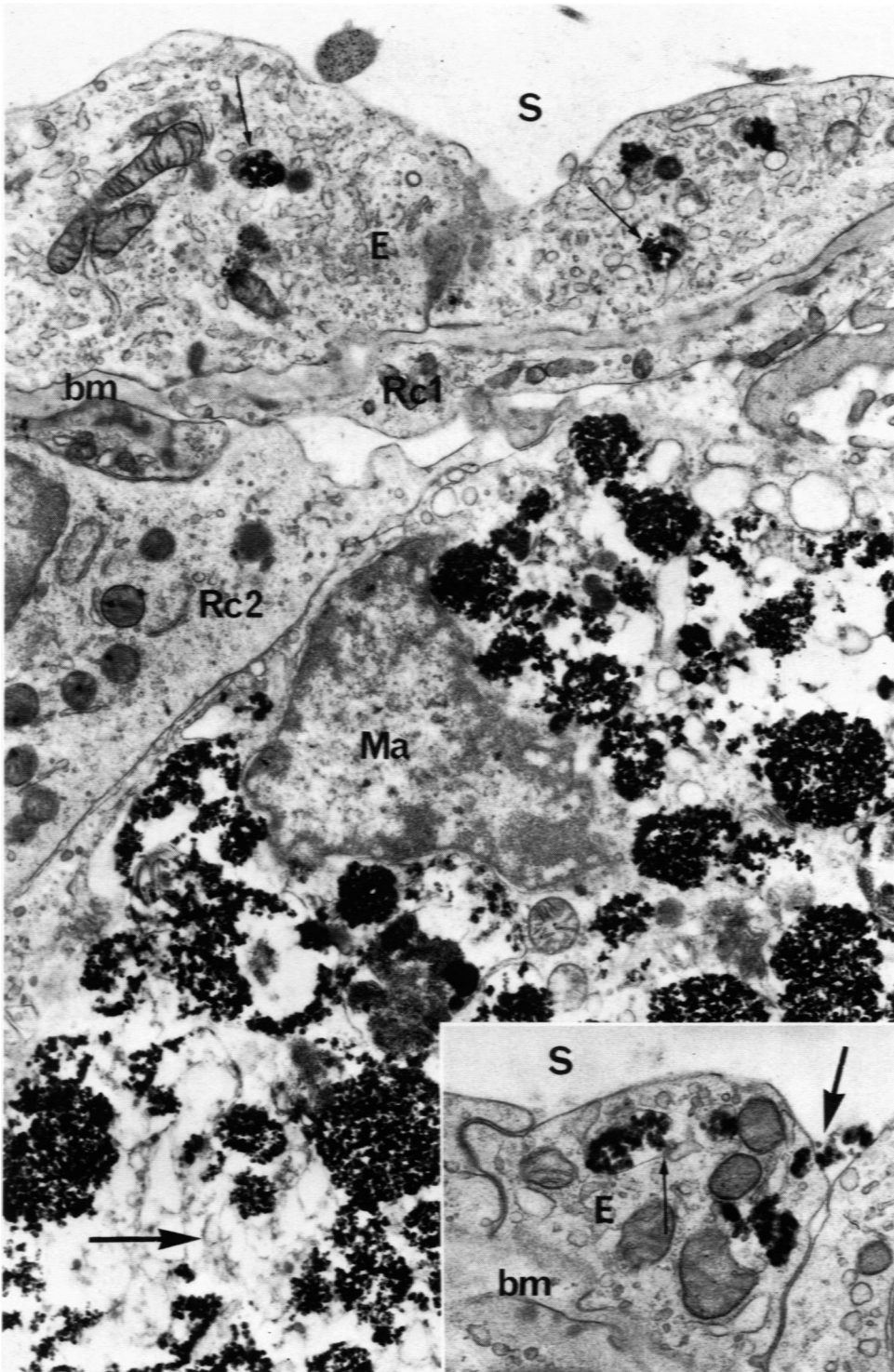


Fig 11. (*upper*) Rabbit spleen; peripheral white pulp. Carbon injection (24 hr). Macrophage (*Ma*) contains large single vacuole (*V*) with carbon particles (*arrow*) at periphery. No other material appears to be present in this inclusion. Macrophage is surrounded by prolongations of reticulum cells (*Rc*) and lymphocytes (*Ly*). Lead hydroxide stain. $\times 8000$.

Fig 12. (*lower*) Rabbit spleen; marginal zone. Carbon injection (1 week). Macrophages (*Ma*) demonstrate vast amount of carbon particles phagocytosed in spleen one week after injection. Carbon lies in vacuoles varying in shape from ovoid to tubular. Lead hydroxide stain. $\times 11,000$.

