Onderstepoort J. vet. Res. 45, 165–186 (1978)

LIGHT AND ELECTRON MICROSCOPICAL STUDIES ON CANINE ENCEPHALITOZOONOSIS: CEREBRAL VASCULITIS

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ABSTRACT

VAN DELLEN, A. F., BOTHA, W. S., BOOMKER, J. & WARNES, W. E. J., 1978. Light and electron microscopical studies on canine encephalitozoonosis: Cerebral Vasculitis. *Onderstepoort Journal of Veterinary Research* 45, 165–186 (1978).

Brain lesions in 2 natural cases of canine encephalitozoonosis were studied by light and electron microscopy. Granulomatous reactions, associated with small vessels and capillaries, partly originated from extensive perithelial cell proliferation which ultimately produced the epithelioid cell component. Diffuse glial reactions apparently occurred in relation to the vasculitis. Lymphoid cells infiltrated the epithelioid and glial cell inflammation. *Encephalitozoon* in all its reproductive stages was identified as the aetiological agent, and ultrastructurally differentiated from *Nosema* on the basis that a single nucleus was observed. Viable organisms were present only within endothelial cells. Macrophages containing dead spores were usually seen around parasitized vessels and, less frequently, in the neuropil. Organisms, whether viable or non-viable, were never seen extracellularly at the ultrastructural level. Selected histochemical stains and electron microscopy were used to differentiate between viable and non-viable spores. Vasculitis is demonstrated as the underlying lesion of canine encephalitozoonosis affecting the brain and is suggested to be the basic factor in the pathogenesis of this disease.

Résumé

ÉTUDES EN MICROSCOPIE OPTIQUE ET ÉLECTRONIQUE SUR L'ENCÉPHALITOZOONOSE CANINE: VASCULITE CÉRÉBRALE

On a étudié en microscopie optique et électronique les lésions cérébrales dans 2 cas spontanés d'encéphalitozoonose canine. Des réactions granulomateuses associées aux petits vaisseaux et aux capillaires avaient leur origine en partie dans une prolifération généralisée de cellules périthéliales, aboutissant à la production des cellules épithélioïdes. Des réactions gliales diffuses se sont apparemment produites en rapport avec la vasculite. Il y avait infiltration de cellules lymphoïdes dans les foyers inflammatoires aux cellules gliales et épithélioïdes.

On a pu identifier Encephalitozoon *dans tous ses stades reproductifs comme étant l'agent étiologique et on a pu le différencier, dans son ultrastructure, de* Nosema *par le fait qu'on n'a observé qu'un seul noyau. On n'a trouvé d'organismes viables qu'à l'intérieur des cellules endothéliales. Des macrophages contenant des spores mortes ont habituellement été observés à l'entour des vaisseaux parasités et, plus rarement, dans le neuropile. Que les organismes fussent viables ou non, l'observation de l'ultrastructure ne les a jamais détectés hors de la cellule. La différenciation entre spores viables et non viables a pu être faite au moyen de colorations histochimiques choisies et du microscope électronique. Il est prouvé que la vasculite est la lésion fondamentale de l'encéphalitozoonose canine qui attaque le cerveau et l'on pense qu'elle est le facteur de base dans la pathogénie de cette maladie.*

INTRODUCTION

The lesions of encephalitozoonosis vary from case to case depending upon the species of animal involved and also, it would seem, upon the species of *Encephalitozoon* which causes the infection. For example, chronic meningo-encephalitis is the prominent manifestation of encephalitozoonosis in mice with little or no clinically apparent sign of infection (Perrin, 1943; Innes, Zeman, Frenkel & Borner, 1962), whereas, in the dog, interstitial nephritis, granulomatous meningo-encephalitis and clinical illness referable to the central nervous system (CNS) are common findings (Plowright, 1952; Basson, McCully & Warnes, 1966; McCully, Van Dellen, Basson & Lawrence, in press).

Vascular involvement in the lesion of encephalitozoonosis has been recorded with most cases since the first aetiological description by Wright & Craighead (1922) of the disease in rabbits. Other reports of this disease in rabbits also record vascular involvement (Oliver, 1922; Malherbe & Munday, 1958; Koller, 1969), including the report in which the aetiological organism was named *Encephalitozoon cuniculi* by Levaditi, Nicolau & Schoen (1923). Furthermore, vascular lesions have been observed in other species of animals, including dogs (Plowright, 1952), cats (Van Rensburg & Du Plessis, 1971), blue foxes (Nordstroga, 1972), and man (Margileth, Strano, Chandra, Neafie, Blum & McCully, 1973). The brain of rabbits and dogs has particularly prominent vascular lesions which range from mild mononuclear perivascular cuffing to haemorrhage, necrosis, granulomatous reaction and the formation of granulomata. Nordstroga (1972) was the first to suggest that the vascular lesion in the blue fox had features in common with periarteritis nodosa. Subsequently, this apparent lesion was also reported in dogs by McCully *et al.* (in press).

In spite of being an obligatory intracellular parasite, the aetiological agent has been frequently reported not only within endothelial and other cells but also as a tightly-packed, round group of organisms apparently free in the neuropil. In addition, the organisms may be found free within the lumen of blood vessels and among inflammatory cells of focal lesions with or without necrosis. Their presence within canine glial cells was reported before their exact identity was known (Kantorowicz & Lewy, 1923). Intraneural occurrence has been described (Wright & Craighead, 1922; Plowright & Yeoman, 1952; McCully *et al.*, in press). Smooth muscle cells of small arterioles may apparently also harbour the organism (Plowright & Yeoman, 1952; Van Rensburg & Du Plessis, 1971; Margileth *et al.*, 1973).

Although several excellent ultrastructural studies of *Encephalitozoon* and other microsporida have been made (Cali, 1970; Sprague & Vernick, 1971), no primary ultrastructural study of the pathological changes seen with encephalitozoonosis has been reported, nor has it been generally recognized that the basic lesion in canine encephalitozoonosis is an

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The views expressed herein are those of the authors and are not to be construed as official or as reflecting the views of the U.S. Air Force or the Department of Defence

Received 30 March 1978—Editor

angeitis. This study combines light and electron microscopic observations of pathological and parasitological changes in the brain, and emphasizes the cellular inflammatory changes affecting small cerebral blood vessels and capillaries. Ultrastructural features of cerebral, parasitic vasculitis are illustrated in detail.

Materials and Methods

A 6-week-old male Toy Pomeranian (Pup A) and an 8-week-old female Boerbull pup (Pup B) from different owners and each exhibiting different clinical signs were presented for postmortem examination. Brain specimens were collected in 10% buffered formalin for histopathology and in 4% (pH 7,25) phosphate buffered glutaraldehyde for electron microscopy. Tissues for histopathology were embedded in paraffin wax, cut at 6 μm thickness, and routinely stained with haematoxylin and eosin (HE). Special straining methods (Luna, 1968) of Giemsa's, Gram's (Humberstone modification), Ziehl-Neelsen's (ZN), Periodic Acid-Shiff (PAS) and Gomori's methanamine silver (GMS) were applied to selected sections. Tissue for electron microscopy was post-fixed in 2% buffered osmium tetroxide, dehydrated in an ethanol gradient and embedded in Epon 812. Sections 1–2 μm thick, cut for tissue orientation and the study of host-parasite relationships, were stained by Chang's method of staining plastic sections with HE (Chang, 1972). Silver sections (60 μm) were cut on a Reichert ultramicrotome, mounted on 200 and 300 mesh copper grids, stained with 1% aqueous uranyl acetate at 60 °C and Reynold's lead citrate at room temperature, and viewed in a Siemens 102 Elmiskop electron microscope (Hayat, 1970; Kay 1965; Pease, 1964). Light microscopic measurements of the parasite were made with a calibrated ocular micrometer.

Results

Clinical observations

Pup A was a 6-week-old male Toy Pomeranian with a history of intermittent nervous signs since the first week of life. These nervous signs were mainly inco-ordination and trembling, with chorea-like movements of the head and neck which caused difficulty with eating. The medial canthus of both eyes was filled with a thick mucopurulent exudate. The puppy which had not been vaccinated against canine distemper, was culled from a breeding kennel because of stunted growth.

Pup B was an 8-week-old female Boerbull pup which had been vaccinated against distemper. When she was presented her gait was unsteady, she walked in circles and was considered partially blind since she would occasionally bump into objects in her path. The pup was thin and her growth unsatisfactory even though she was fed an excellent ration and was free of internal parasites. Blood smear examination was negative for haemoparasites. For necropsy examination both pups were euthanized with 1 g sodium pentobarbital administered intravenously.

Gross pathology

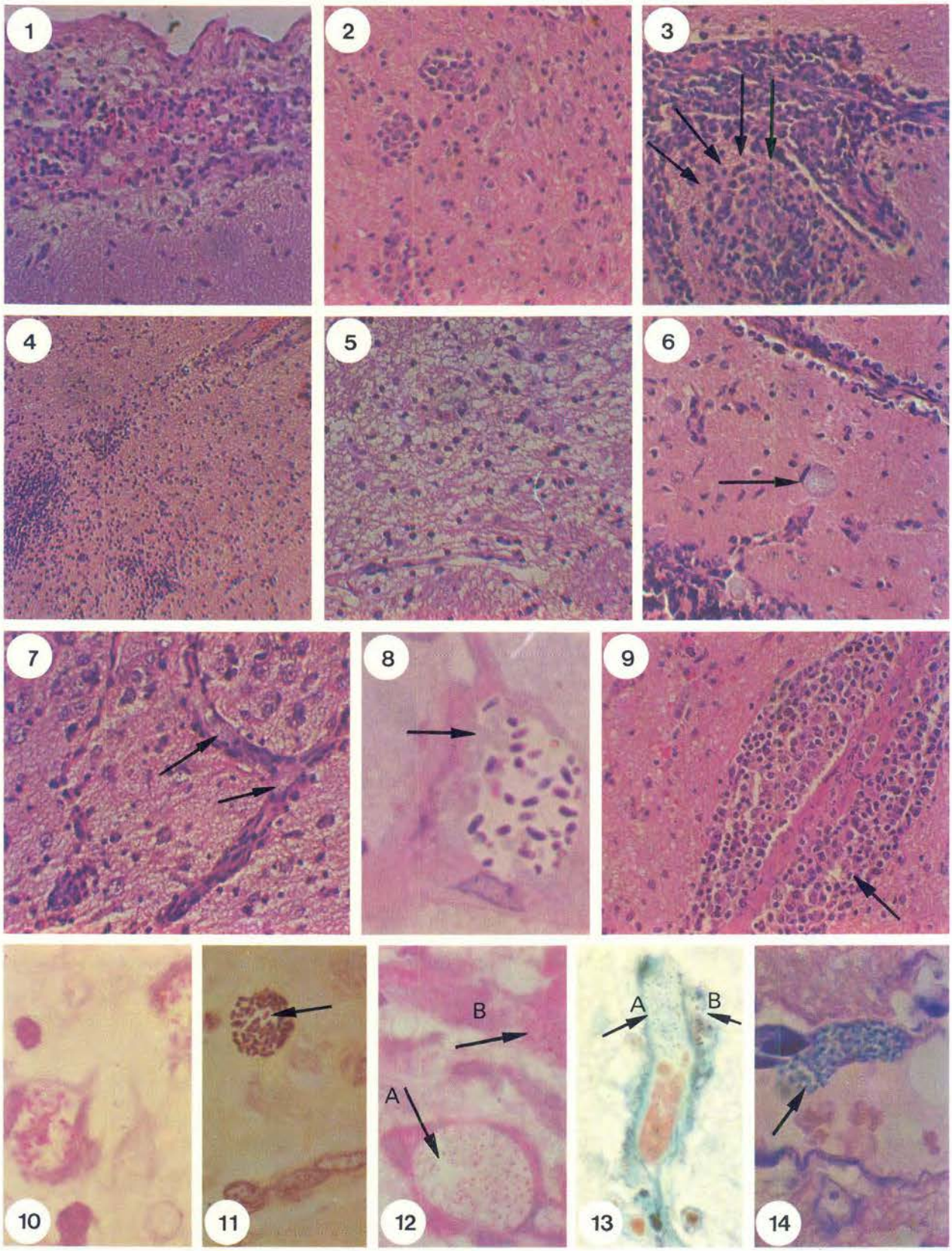
No notable change of the brain of either Pup A or Pup B was seen at necropsy. Visual inspection revealed an apparently normal brain.

Histopathology

Pup A

Pathological changes were acute to subacute, with lesions present at all levels of the brain and no apparent predilection for any particular area. A moderately severe, multifocal to diffuse meningoencephalitis was present in all sections examined. Meningitis was typified by lymphoid infiltrates and adventitial hyperplasia seen over gyri (Fig. 1), often more severe in the depths of sulci. Small vessels and capillaries in the neuropil appeared prominent when affected with adventitial hyperplasia and hypertrophy. Small cuffs of lymphoid cells often surrounded this early vascular reaction (Fig. 2). Cells of the adventitia developed an epithelioid appearance, with mononuclear cells infiltrating among them. This combined cellular inflammation usually formed a perivascular cuff and created a nodular, granulomatous perivasculitis (Fig. 3). Focal glial hyperplasia, mostly astrocytes with some microglia, circumscribed small vessels with adventitial hyperplasia, and a more diffuse gliosis surrounded both of these apparently interdependent cellular reactions (Fig. 4). Diffuse astrocytic proliferation and microglial infiltration affected both

FIG. 1 Cerebrum, gyrus. Lymphoid infiltrates and adventitial hyperplasia characterize focal meningitis. H & E ×300
FIG. 2 Neuropil. Small cuff of lymphoid cells, mostly plasmacytes, surrounding small vessels and capillaries affected by adventitial hyperplasia. H & E ×300
FIG. 3 Cerebellum. Granulomatous vasculitis with mononuclear cell infiltration among epithelioid cell proliferation. This vasculitis has a nodular appearance when cut in cross section (arrows). H & E ×300
FIG. 4 Midbrain. Focal glial hyperplasia and lymphoid infiltrates circumscribe small vessels with adventitial hyperplasia. Density of diffuse gliosis surrounding this cellular inflammation decreases as the distance from affected vessel increases. H & E ×150
FIG. 5 Cerebrum, white matter. Diffuse gliosis with moderate to severe status spongiosus. H & E ×300
FIG. 6 Cerebellum. Group of *Encephalitozoon* (arrow) within a capillary endothelial cell. No inflammatory reaction against them is apparent. Organisms stain poorly with routine H & E ×300
FIG. 7 Cerebrum. Group of intra-endothelial *Encephalitozoon* (arrows). Adventitial reaction of an affected vessel appears to be the first morphological evidence of response to infection. Organisms are often difficult to find if they are within endothelial cells of vessels with inflammatory reaction. H & E ×300
FIG. 8 Cerebellum, 1–2 μm plastic section, Epon. Cytoplasmic sporogony vacuole within endothelial cell. Mature spores are densely basophilic; immature spores tend to be eosinophilic. Large greyish structures in periphery of vacuole are sporonts undergoing binary fission (arrow). H & E ×2 000
FIG. 9 Cerebellum. Granulomatous, nodular vasculitis is prominent in subacute forms of the disease. Hyperplastic perithelial cells transforming into epithelioid cells (arrow) are infiltrated by lymphoid mononuclear cells. H & E ×300
FIG. 10 Brain. Acid-fast group of organisms within endothelial cells. ZN ×1 125
FIG. 11 Brain. Group of Gram-stained spores within an endothelial cell. The majority of these spores are Gram-positive and probably mature; some are Gram-negative (one at arrow) and thought to be immature. Humberstone ×1 125
FIG. 12 Brain. Viable organisms within an endothelial cell (A) reveal a small reddish point at their anterior pole when stained with PAS. Non-viable organisms within a macrophage (B) are also PAS positive, but with these the PAS mass is larger and surrounded by an artifactual clear space. PAS ×1 125
FIG. 13 Brain. The GMS pattern of staining is identical with that of the PAS. Viable organisms within an endothelial cell (A) are revealed by a small black point over their anterior vacuole. Larger GMS-positive masses, usually surrounded by a "halo", are non-viable organisms within a perithelial cell macrophage (B). GMS ×1 125
FIG. 14 Brain. Giemsa's stains viable organisms within endothelial cells well. Here they are seen being released into the vascular lumen (arrow). Non-viable organisms within macrophages do not stain well with Giemsa's stain. ×1 125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grey and white matter, but was often more severe in the white matter where they were frequently accompanied by some degree of status spongiosus (Fig. 5). Fibrinoid, necrotic vasculitis occasionally affected a meningeal vessel. Haemorrhage was rarely observed and thrombosis not at all. The spinal cord was not examined.

Tightly-packed, focal groups of organisms were easily seen with HE stain, even when no cellular inflammatory reaction coincided. They appeared as pale, greyish, round to oval masses (Fig. 6) and were found in both grey and white matter with apparent equal frequency. Their intra-endothelial cell position was not readily determined with each group. However, similar groups in areas of vasculitis and perivasculitis were easily recognized within endothelial cells (Fig. 7). Individual spores measured on the average $1,5 \times 2,5$ μm while a larger stage of the organism, the dividing sporont, as seen on the periphery of groups embedded in 2 μm thick plastic sections, measured about $2,5 \times 4,5$ μm (Fig. 8). Mature spores were acid-fast (Fig. 10), Gram-positive (Fig. 11), parts of them PAS- (Fig. 12) and GMS-positive (Fig. 13), and they stained well with Giemsa's stain (Fig. 14). The sporont, and immature stage, was Gram-negative and not acid-fast. Also, some spores were not acid-fast, tended to be Gram-negative, and were therefore thought to be immature. In addition, some spores polarized with the use of polarizing filters. The mature spore possessed a distinct, though very small, PAS-positive reaction which coincided with the polar cap and was seen as a reddish point over the anterior vacuole (Fig. 12). A slightly larger black point in the same location was observed with the GMS stain (Fig. 13). These specific histochemical reactions were obtained with viable, mature spores within endothelial cells and were in contradistinction to the PAS and GMS reaction on apparently non-viable spores not within endothelial cells. The latter spores were very numerous and found within adventitial or perithelial cell macrophages surrounding cerebral vessels (Fig. 12 & 13) and, rarely, within glial macrophages away from the vasculature. These spores were not Gram-positive or acid-fast and did not stain well with Giemsa's. They could be recognized as spores by their size, shape, and translucent space which was seen as a halo (Fig. 12) surrounding an internal reddish (PAS) or black (GMS) homogeneous mass. No differentiation of the polar cap with PAS or GMS stain was discernible on these spores within macrophages. Later, with the aid of electron microscopy, it was confirmed that viable spores with discernible organellae were present only within endothelial cells, whereas non-viable spores with no organellae remaining were seen only within macrophages.

Pup B

Even though the clinical presentation was quite different, histopathological changes in the brain were basically similar to those found in Pup A. Lesions, however, were of a more subacute nature. In this case of severe meningoencephalomyelitis, the most prominent feature was extensive focal granulomatous reaction usually observed around small vessels. All levels of the brain and spinal cord had lesions, with the distinction that significantly large areas of midbrain and cerebellum were free of morphological change. Small vessels in the neuropil were affected with a severe adventitial reaction which eventually formed large cuffs of epithelioid cells. Large numbers of mononuclear cells, which included mostly plasma cells, some lymphocytes, and rarely microglia, infiltrated these cuffs and together formed a typical perivascular nodule which was far more prominent than in Pup A (Fig. 9). Reactive astrocytes and infiltrating microglia were diffusely scattered near and surrounding these nodules. Necrosis of vessels was rarely seen and perivascular cuffing with mononuclear cells only was not common.

Observations on the organism as described for Pup A are applicable to Pup B. However, endothelial cells containing viable, mature spores were not nearly as numerous, and Gram-positive as well as Giemsa's staining organisms were not easily found. Conversely, PAS and GMS positive spores within macrophages were more numerous and definitely easier to find than Gram-positive spores.

Ultrastructural observation

Unless stated otherwise, ultrastructural observations apply to the lesions and organisms in the brain of both Pup A and Pup B.

The aetiological agent, *Encephalitozoon*, was seen in 5 stages of its reproductive life cycle. The first and earliest stage observed was free within host cytoplasm and usually appeared as multiple, uninuclear parasites (Fig. 15). They seemed to form peripheral to what was destined to become an eccentric, cytoplasmic sporogony vacuole. Usually, when the unit membrane of this vegetative stage was seen in contact with the developing sporogony vacuole, it was thicker and more electron dense. This membrane development apparently proceeds until the organism is completely detached from the host cytoplasm and becomes free within the periphery of the sporogony vacuole (Fig. 16). It is during this the first phase of development, that the vegetative stage transforms into a sporont. Binary fission of the sporont, the second stage, occurs in the periphery of a sporogony vacuole (Fig. 17), to produce 2 daughter cells, the sporoblasts. Sporoblasts, the third stage, move towards the centre of the vacuole where they elongate and develop into immature spores, the fourth stage, and finally into mature spores, the fifth stage (Fig. 18).

Distinctive features served readily to identify each stage. Irregularly round, uninuclear, vegetative stages were the only stage completely within and surrounded by host cytoplasm. The mechanism of proliferation was not determined. Binary fission was never observed. Sporonts were still partly attached to host cytoplasm and partly within the periphery of a sporogony vacuole. Their unit membrane was in the process of becoming more electron dense and thicker. They were round to elongate, usually seen in the process of binary fission and often revealed with 2 nuclei, 1 for each daughter cell. Sporoblasts had a single nucleus, and a conspicuous electron dense unit membrane. They also revealed the development of primordial organellae such as filament genesis. Immature spores revealed their coiled filament clearly, and their thickening cell wall, its trilaminar nature still discernible, was not as electron dense as that of a mature spore (Fig. 19). Mature spores were usually so dense that internal organellae were difficult to observe, although a coiled filament and sometimes a single nucleus were seen. A prominent, distinguishing feature of the mature spore was its cell wall of about 300 Å which included 225 Å electron translucent space just peripheral to the parasite's cytoplasmic membrane (Fig. 18 & 19). This clear space was very unstable under the electron beam and was the site of large artifactual tears or holes within sections when large numbers of spores were cut.

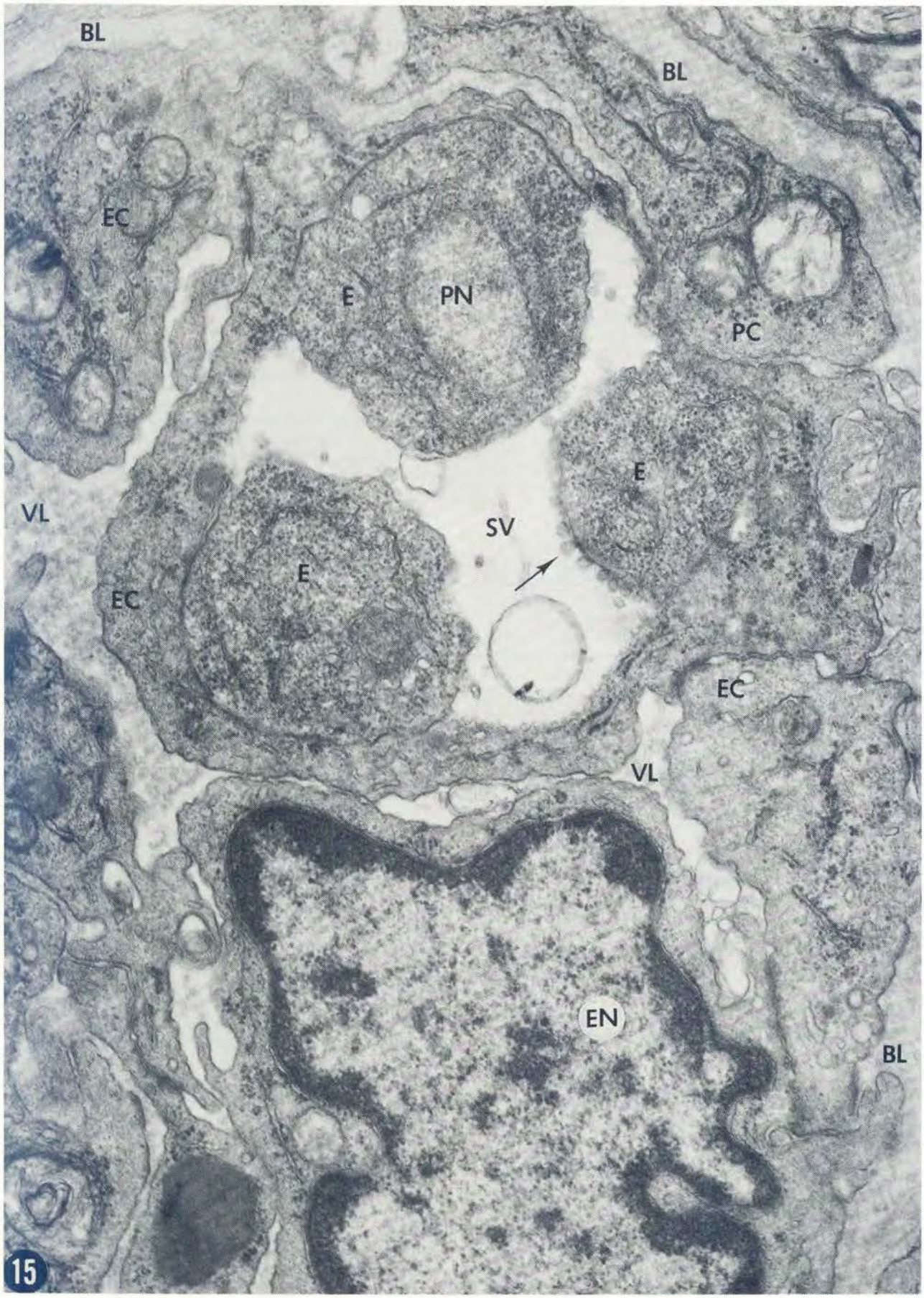


FIG. 15 Parasitized endothelial cell (EC). Three parasites (E) in their late vegetative stage are revealed partly projected into the periphery of a developing sporogony vacuole (SV) while still attached to the host cell cytoplasm. Remnant elements of host cell cytoplasm are loose in the vacuole and attached to the free border of the parasite (arrow). EN = endothelial cell nucleus; VL = vessel lumen; PN = parasite nucleus; BL = basement lamina; PC = cytoplasmic process of a perithelial cell. ×35 000

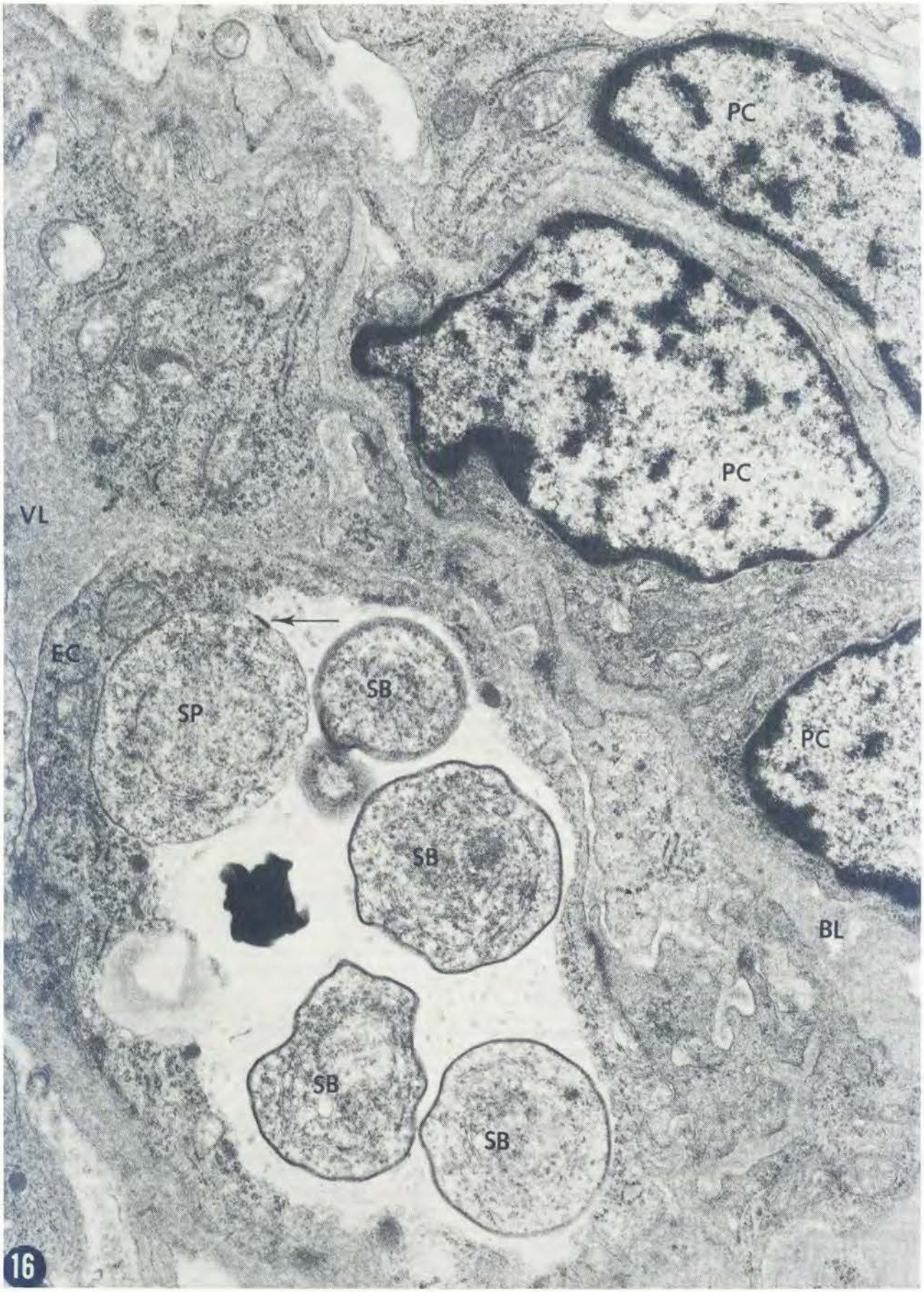
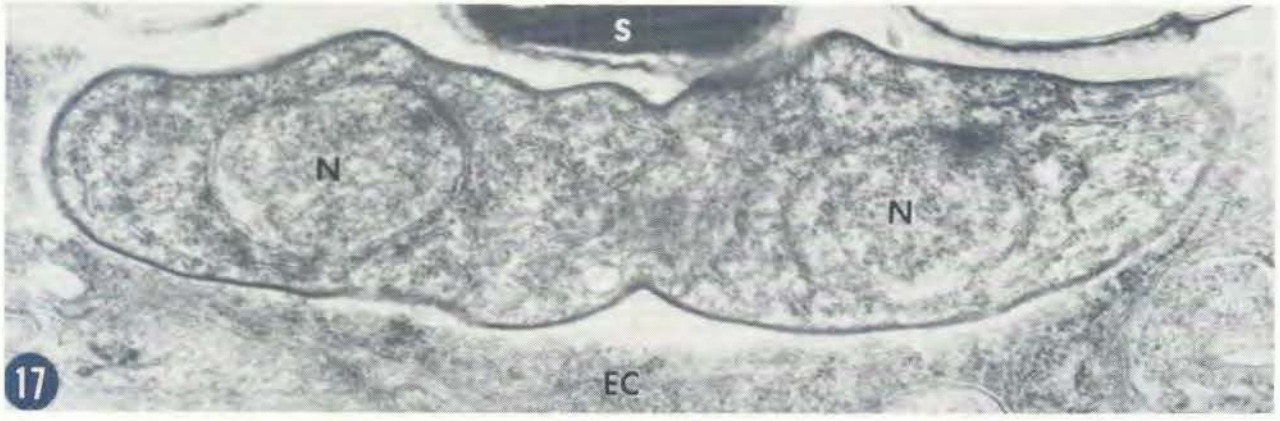


FIG. 16 Parasitized endothelial cell (EC) with surrounding reactive pericytes (PC). Four sporoblasts (SB) are recognizable from their dense limiting membrane and are entirely free within the sporogony vacuole. The organism (SP), still partially attached to host cytoplasm, appears to be a late vegetative stage developing into a sporont. The density (arrow) of its limiting membrane has begun to increase (arrow). ×17 500



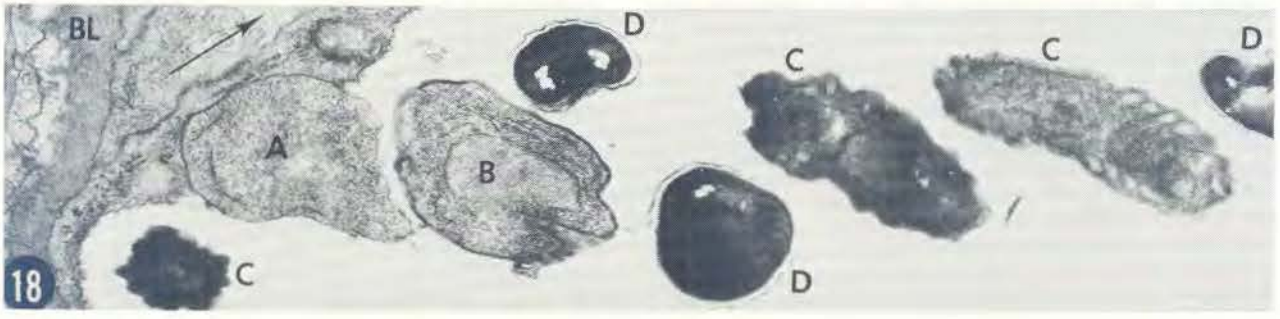


FIG. 17 Dividing sporont in periphery of sporogony vacuole. A single nucleus is provided for each daughter cell which continues to develop as a sporoblast. The sporont's unit membrane is more electron-dense and thicker than that of the previous vegetative stage. N = nucleus; S = spore; EC = endothelial cell cytoplasm. ×37 500

FIG. 18 Stages seen in the reproductive life cycle of *Encephalitozoon*. All are in a sporogony vacuole except the late vegetative or early sporont stage (A) which is in an intermediate zone between vacuole and host cytoplasm. The sporoblast (B) has a conspicuous electron-dense, limiting membrane but no clear evidence of a filament. Immature spores (C) are overall more electron-dense, are often crenated (undoubtedly a shrinkage artifact) and reveal part of a coiled filament. Mature spores (D) are very electron-dense and are surrounded by a clear space (halo), part of which is considered a shrinkage artifact, within their wall. BL = basement lamina; arrow: vessel lumen. ×21 000

The sporogony vacuole with viable organisms in different stages of development was seen only within endothelial cells. When the vacuole was fully distended with organisms, only a thin strand of cytoplasm separated spores from the vascular lumen (Fig. 19). A small vessel or capillary often had only 1 of its endothelial cells parasitized. Such a site may be entirely free of surrounding inflammatory cells or even reactive, hypertrophied perithelial cells (Fig. 20).

The first morphological evidence we saw of damage to the vasculature was a non-cellular, oedematous inflammatory change, indicative of increased vascular permeability (Fig. 21). Perivascular astrocytic foot processes may become dilated (Fig. 22). The basement lamina became swollen and appeared frayed and loose, and widened interstitial spaces of the neuropil in the vicinity of a vessel so affected usually indicated mild perivascular oedema (Fig. 21). Interstitial oedema was also observed focally in severe proportions at all levels of the brain (Fig. 23), especially in the brain of Pup A.

Severe ultrastructural change was observed as an extensive cellular inflammatory reaction directly or indirectly affecting vessels that were or had been parasitized. Lymphocytic infiltrates accumulated in dilated perivascular spaces (Fig. 24) and they percolated through brain interstitial spaces as they migrated towards or away from vessels (Fig. 25). Lymphocytes and plasmacytes infiltrated among perithelial cell processes (Fig. 26), and also peripheral to perithelial cells, transforming them into a nodule of epithelioid cells (Fig. 27). A macrophage's cellular process was often seen surrounding a large part of the epithelioid cell reaction (Fig. 27 & 29). Concomitantly, endothelial cells and perithelial cells became hypertrophied. Within perithelial cells especially, but also within endothelial cells, lysosomal activation was very prominent (Fig. 28). From these acute to subacute inflammatory changes, the lesion developed into subacute to chronic dimensions. There was rank proliferation of perithelial cells which were eventually transformed into epithelioid cells as they accumulated into a multilayered cuff around parasitized vessels (Fig. 29). This constitutes the major cellular component of the granulomatous reaction seen with the light microscope.

Spores were never seen extracellularly. When spores were not within endothelial cells they were always non-viable and phagocytized by a macrophage. Non-viable spores were readily identified by their size and the characteristic clear space seen between a remnant of the parasite's cell wall, recognized as a thin electron dense line, and its cytoplasmic membrane which was no longer discernible (Fig. 30, 31 & 32). This clear space appears to coincide with the electron translucent space described above for mature, viable spores. No recognizable organellae, including the cytoplasmic membrane, remained in non-viable spores, all having been digested to a moderately electron-dense homogeneous mass.

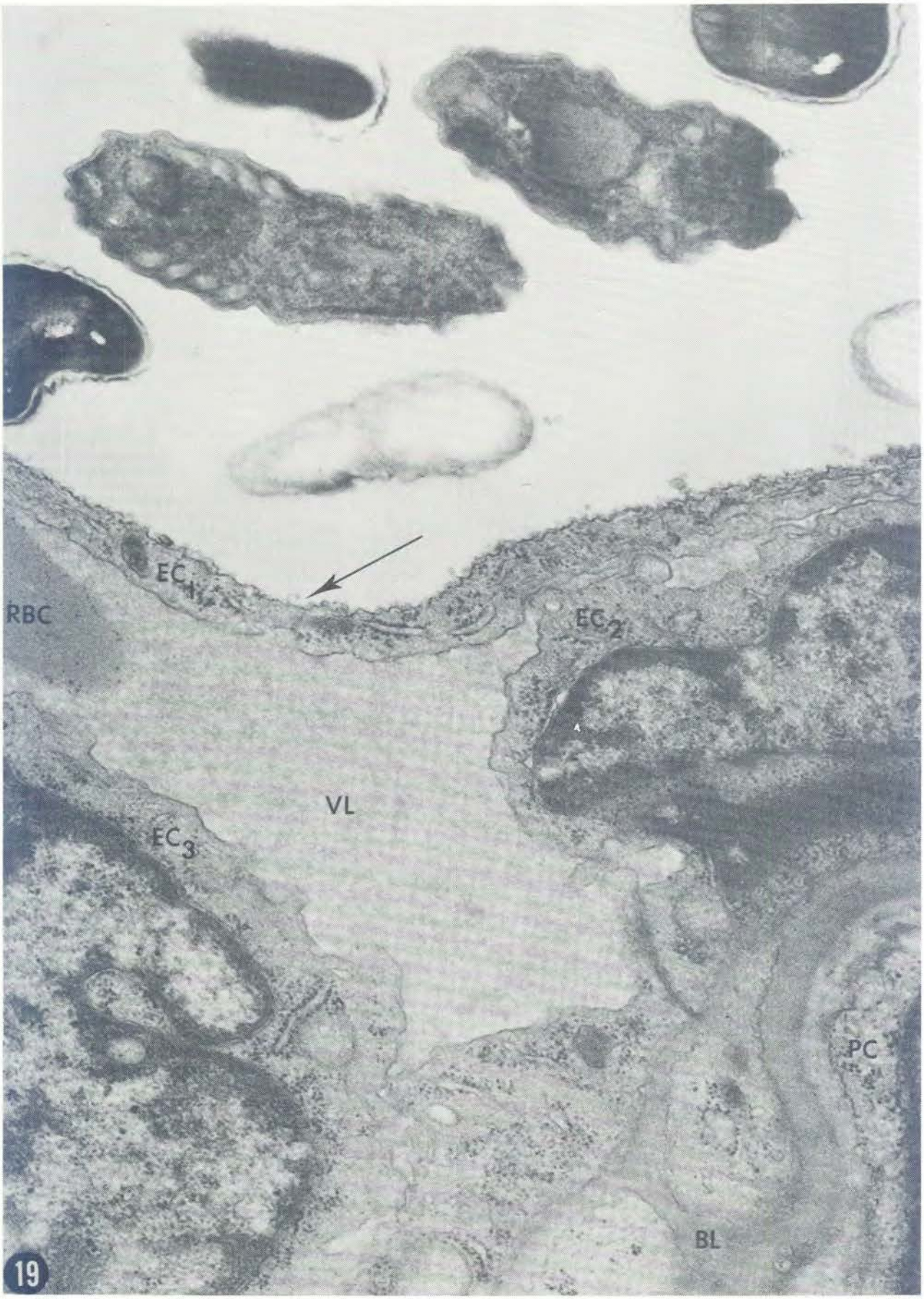


FIG. 19 Spores in a distended sporogony vacuole ready to be released into a patent vascular lumen (VL). It appears that rupture (arrow) of the parasitized endothelial cell (EC_1) is imminent. RBC = red blood cell; $EC_{2\ \&\ 3}$ = non-parasitized endothelial cell; PC = perithelial cell; BL = basement lamina. ×35 000

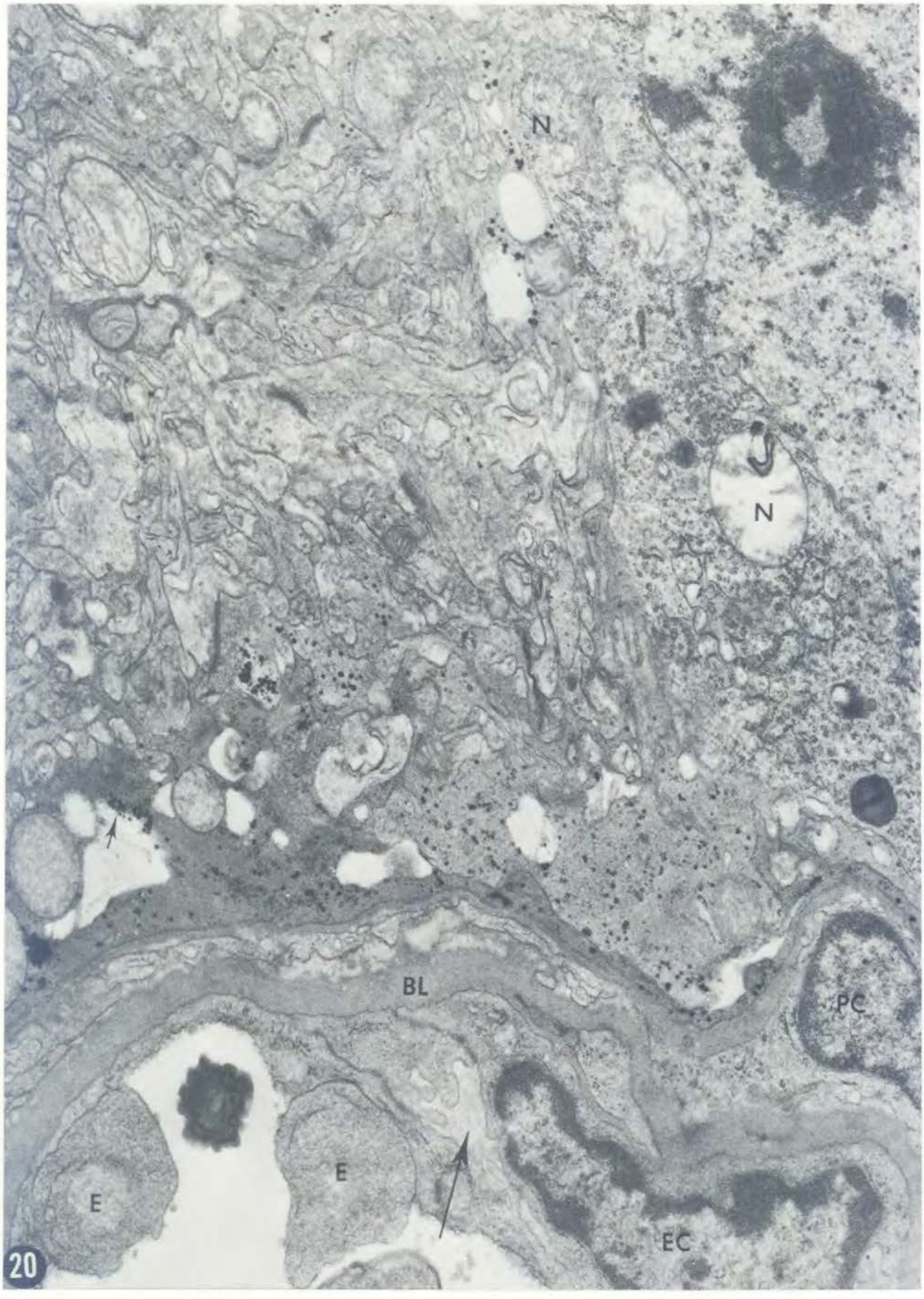


FIG. 20 Ultrastructurally normal neuropil with neuron in juxtaposition to a parasitized capillary. Dense black foci are overstained glycogen particles (small arrow). The small clear spaces in the neuropil immediately surrounding the vessel may indicate early perivascular oedema, but there is no evidence of cellular inflammation. N = neuron; PC = perithelial cell; EC = endothelial cell; E = *Encephalitozoon*; BL = basement lamina; large arrow = vascular lumen. ×21 000

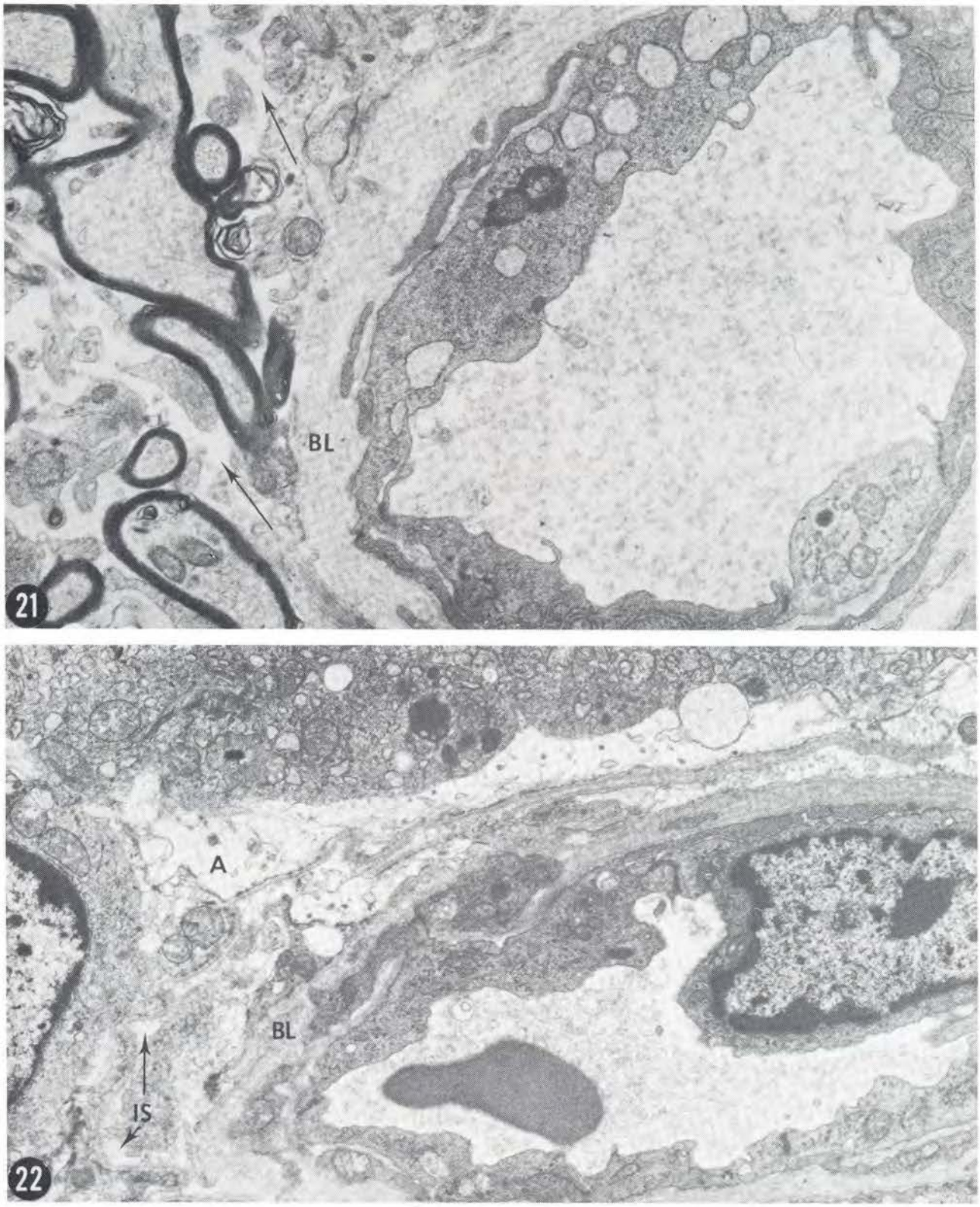


FIG. 21 A non-parasitized capillary. Morphological evidence of increased vascular permeability is seen in a swollen, frayed basement lamina (BL) and enlarged spaces of the neuropil interstitium (arrows). ×14 000

FIG. 22 Swollen astrocytic foot process (A), dilated interstitial spaces (IS), and swollen, disintegrating basement lamina (BL) indicate early vascular impairment resulting in increased permeability. ×13 000

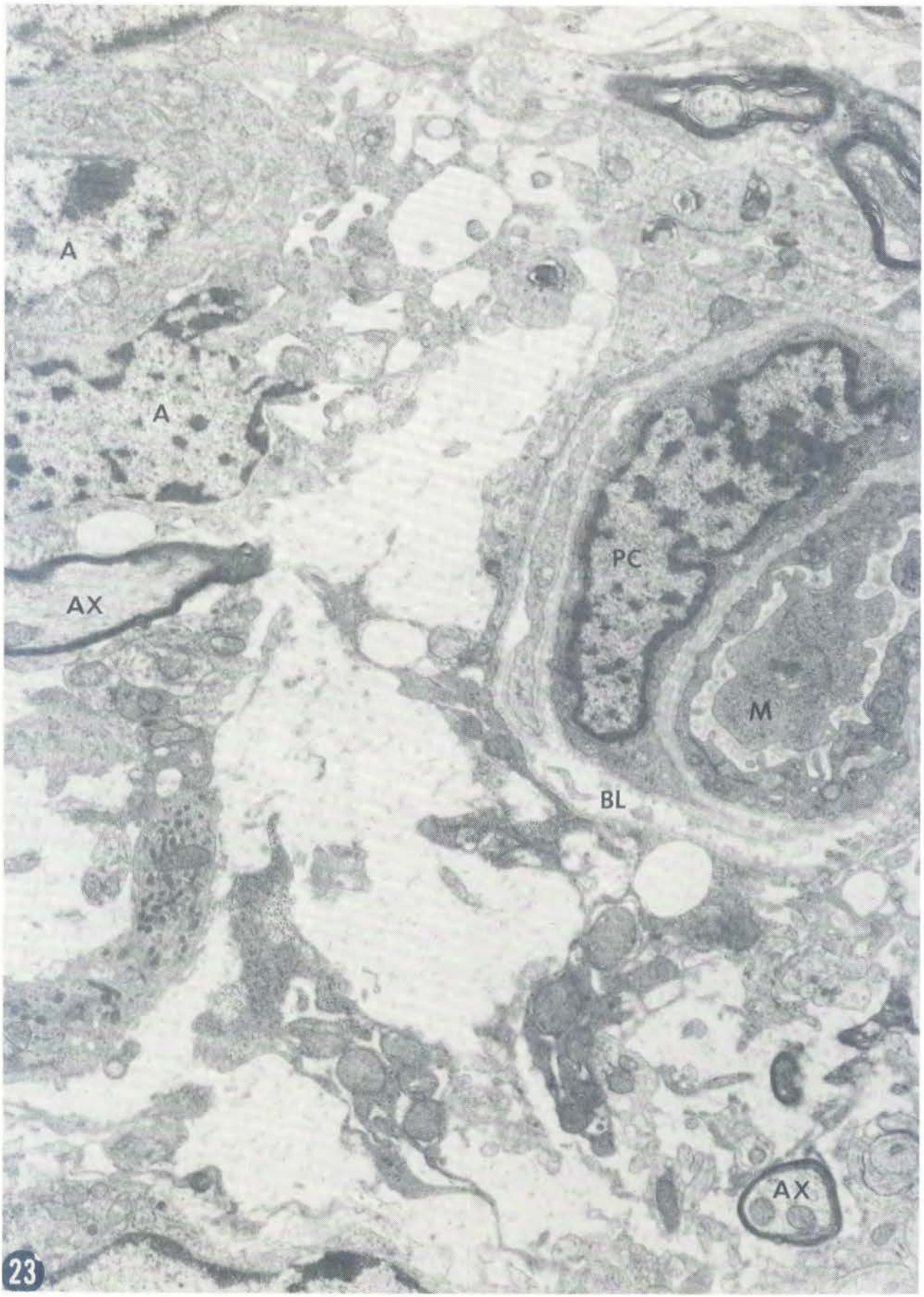


FIG. 23 Large clear spaces interspersed with strands of neuropil are indicative of severe interstitial oedema and can be found anywhere in the brain of acute cases. Astrocytes (A) and perithelial cells (PC) are reactive in the area. Some axons (AX) remain unaffected by the inflammation. A circulating macrophage (M) is seen in a capillary. BL = basement lamina. ×10 500

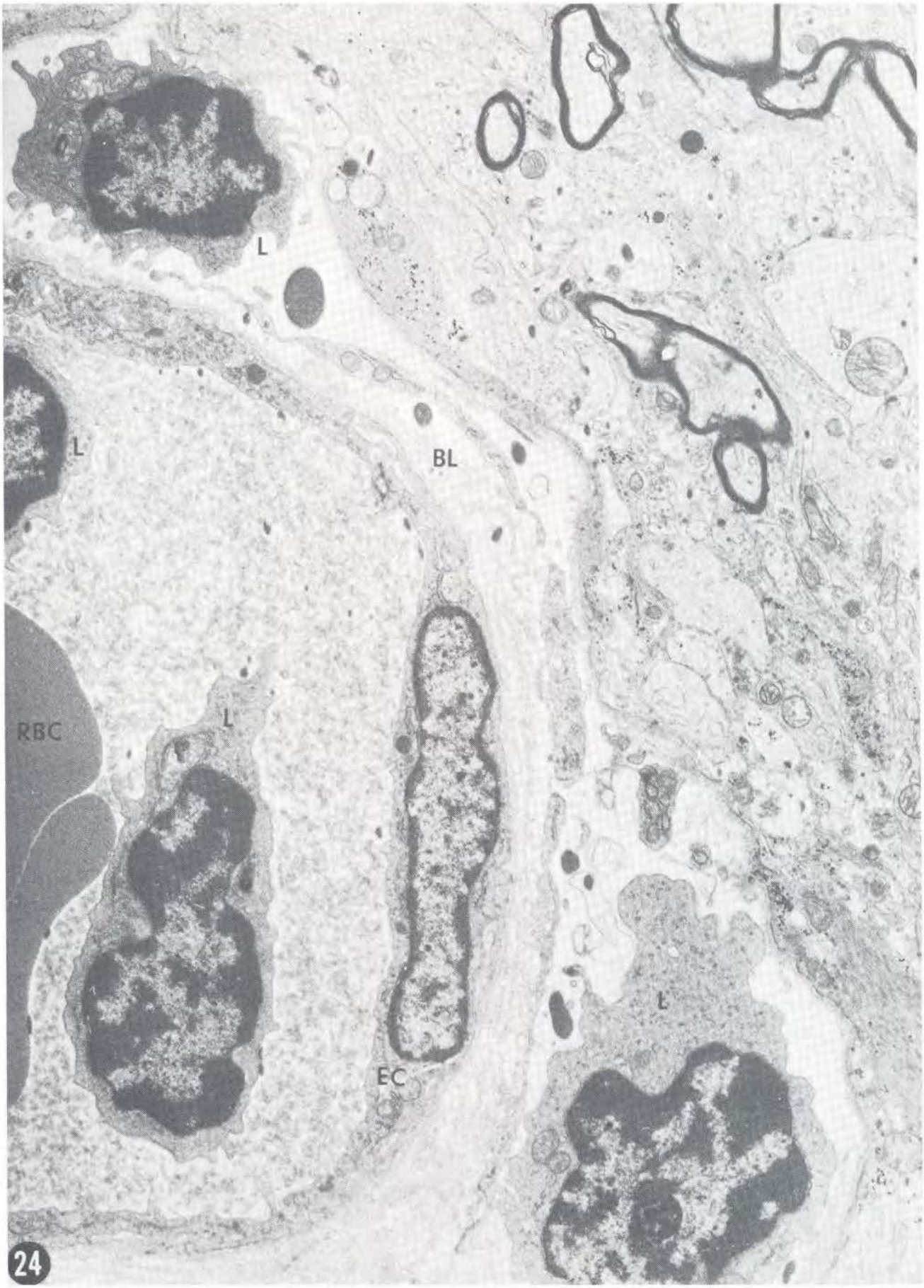


FIG. 24 From the general circulation, lymphocytes (L) accumulate in dilated perivascular spaces. Basement lamina (BL) appears swollen and frayed, but adjacent neuropil appears relatively normal. RBC = red blood cells; EC = endothelial cell. ×10 500

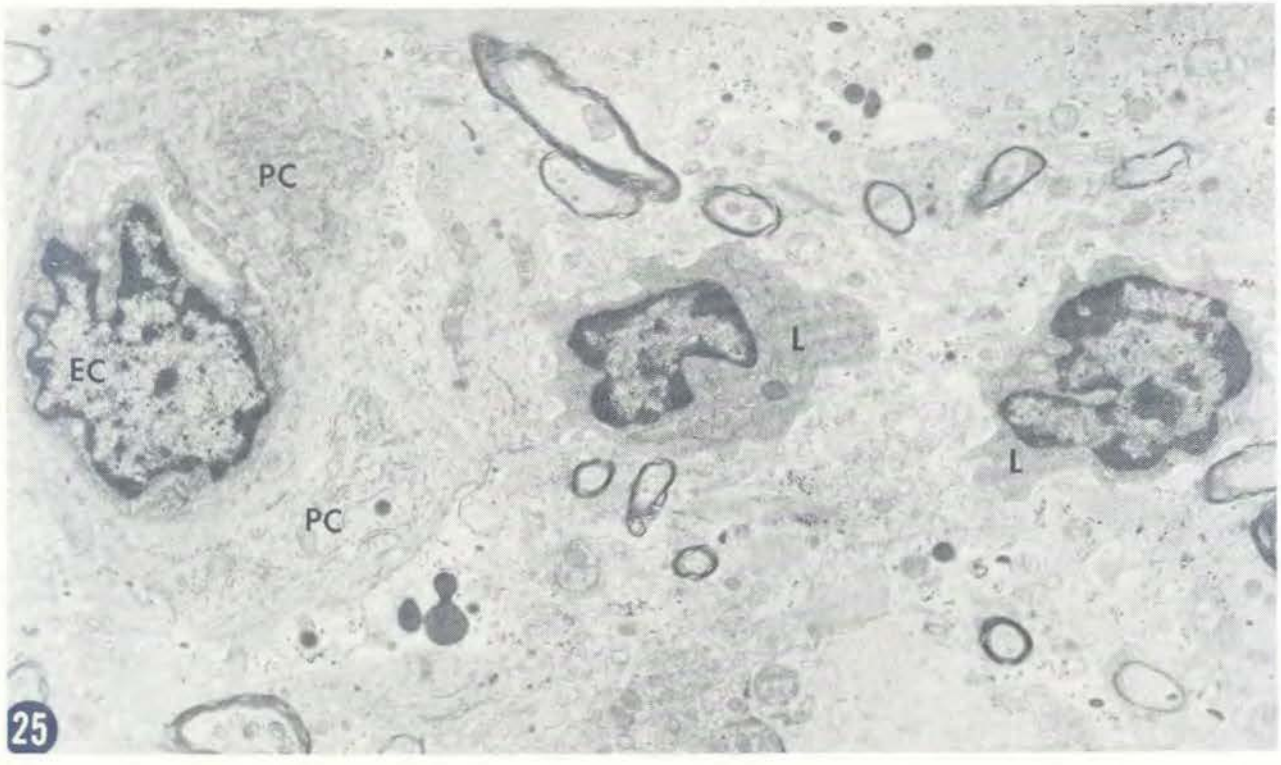


FIG. 25 Lymphocytes (L) percolate through the neuropil towards or away from capillaries. Cytoplasmic processes of reactive perithelial cells (PC) are surrounding the capillary. EC = endothelial cell. ×7 500

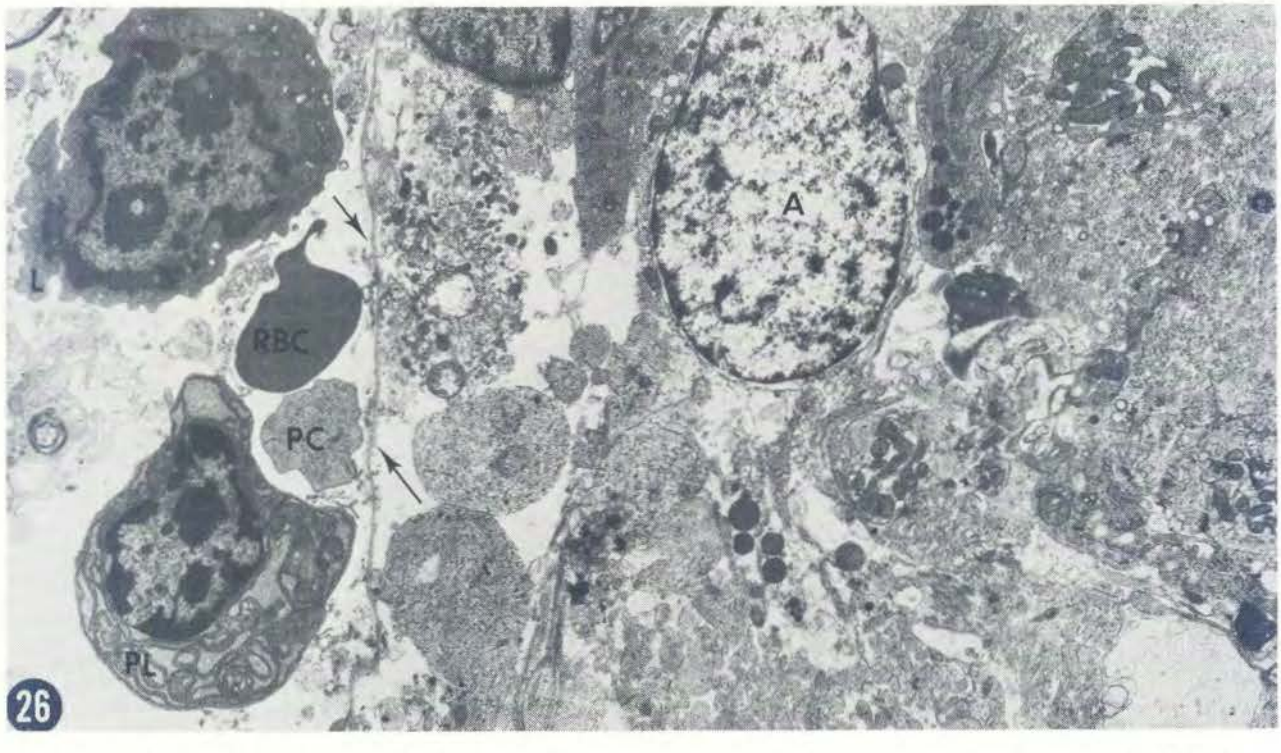


FIG. 26 Lymphocytes (L) and plasmacytes (PL) are infiltrating among perithelial cell processes (PC & arrows). Red blood cells (RBC) are occasionally seen along with them, while astrocytes (A) stand by peripherally. ×7 500

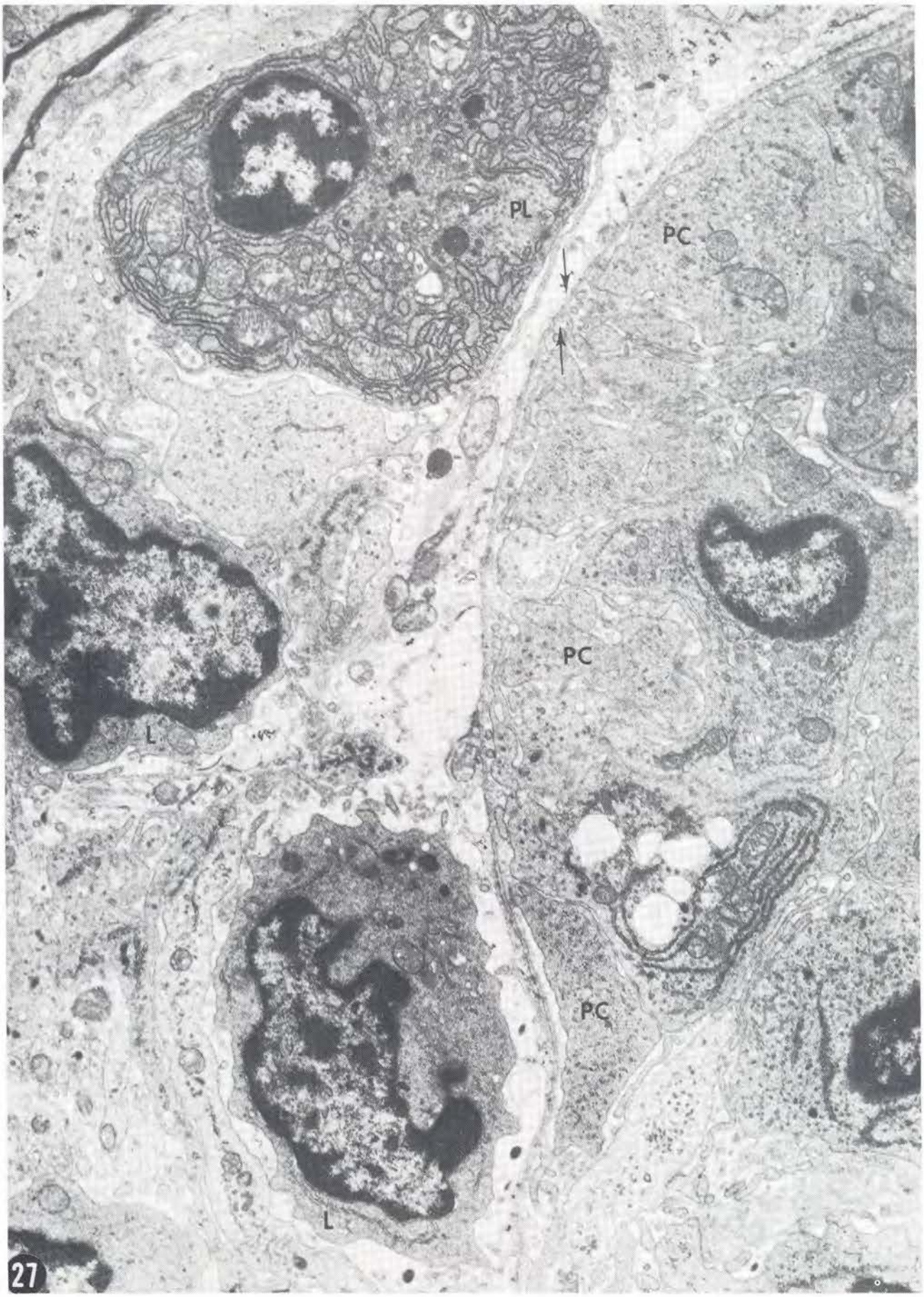


FIG. 27 Lymphocytes (L) and plasmacytes (PL) accumulate in an outer zone around a cuff of proliferating perithelial cells (PC) which transform into a nodule of epithelioid cells. A thin macrophage or perithelial cell process surrounds the nodular inflammation (arrows). This is a typical nodular inflammatory cell reaction around parasitized vessels. ×10 500

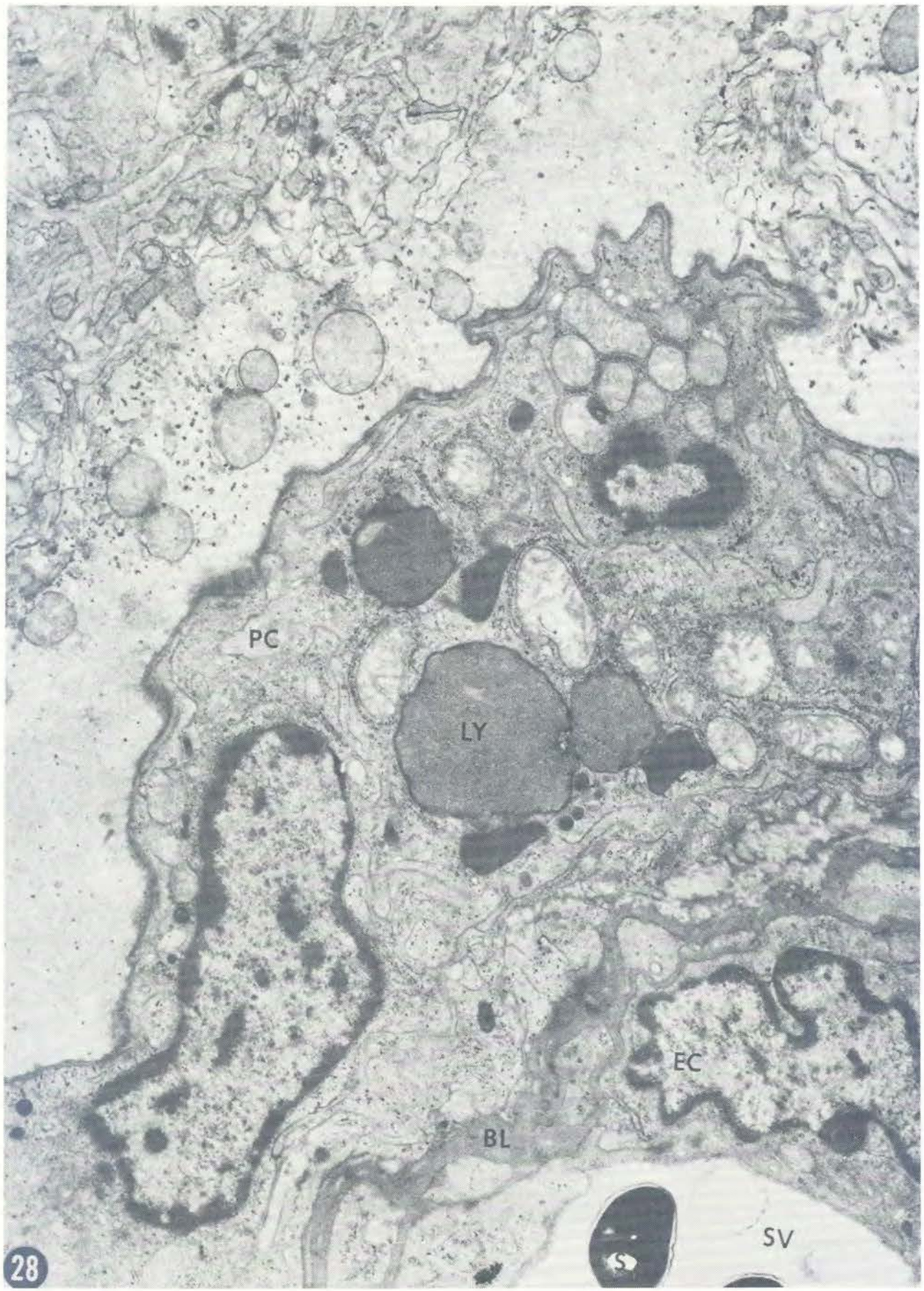


FIG. 28 Hypertrophied perithelial cell (PC) with prominent activated lysosomes (LY) surrounds a parasitized capillary. BL = basement lamina; EC = endothelial cell; S = spore; SV = sporogony vacuole. ×14 000

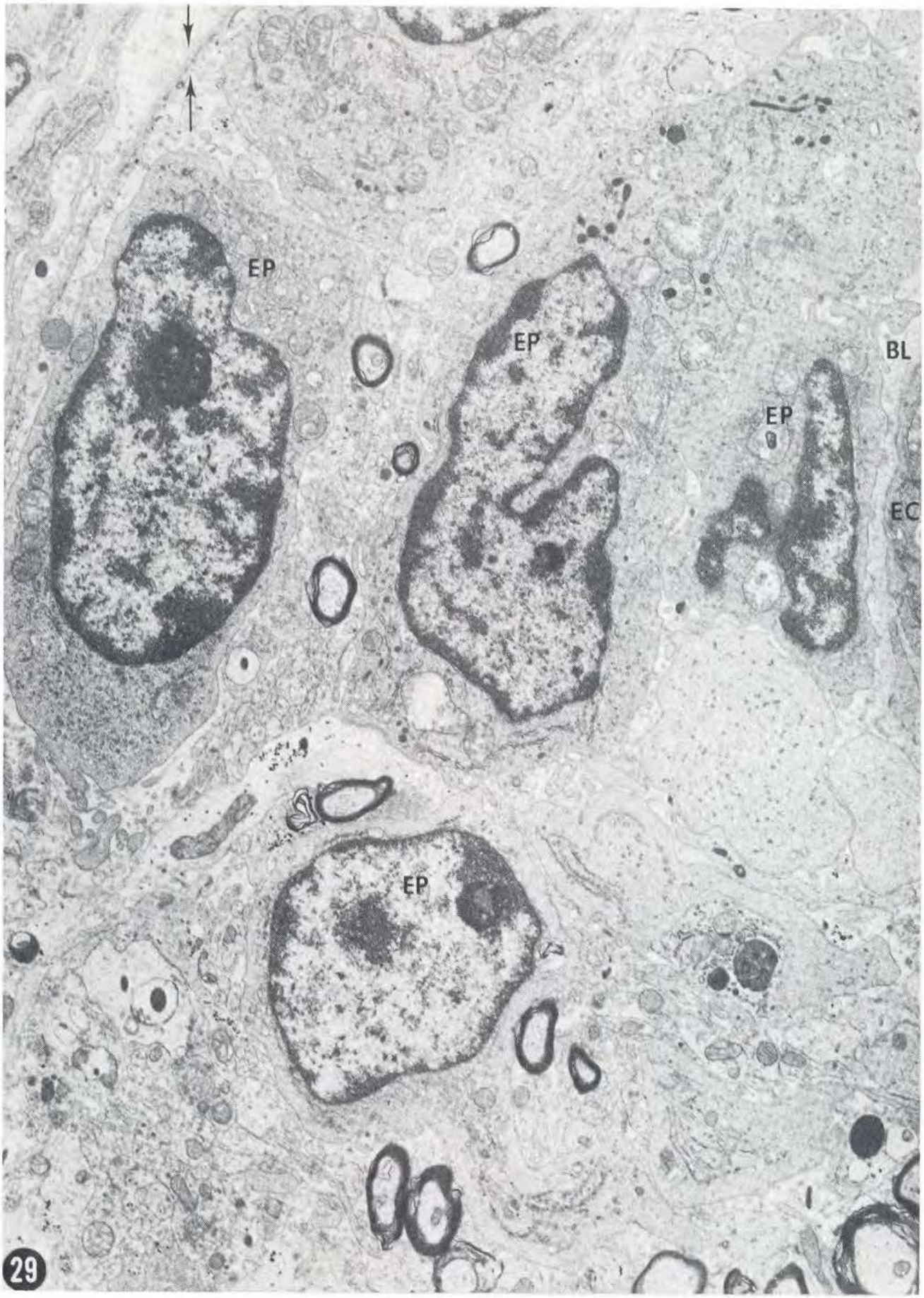
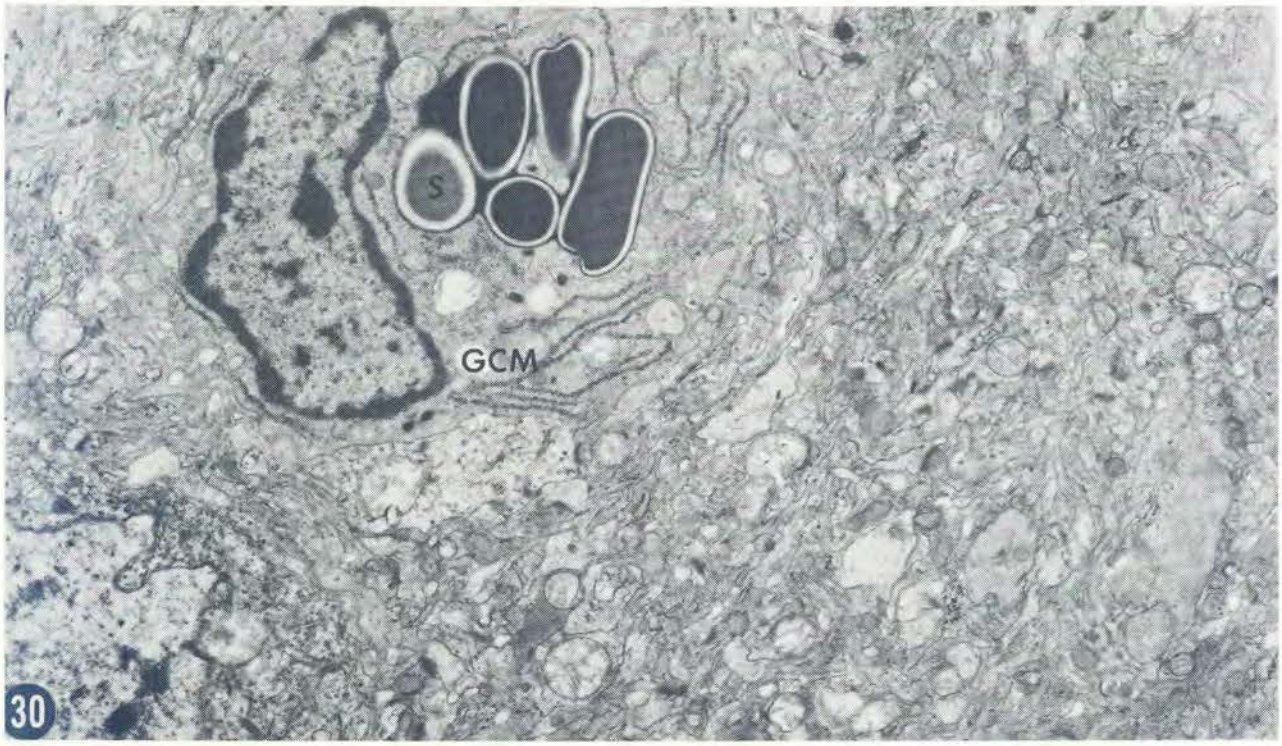


FIG. 29 Proliferated perithelial cells have transformed into a three-layered cuff of epithelioid cells (EP) around a parasitized vessel. A thin macrophage process surrounds this nodular inflammatory reaction (arrows). This cellular inflammation is typical of the granulomatous vasculitis prominent in subacute cases. EC = endothelial cell; BL = basement lamina. ×10 500



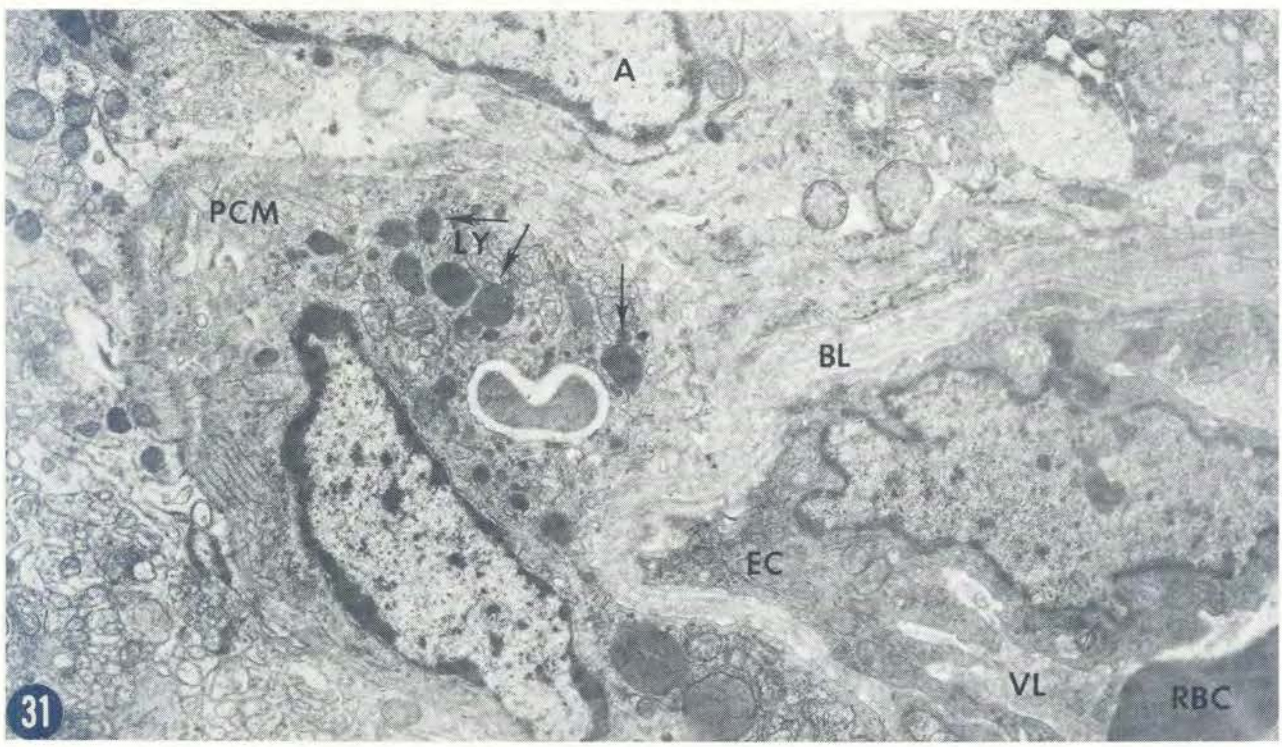


FIG. 30 Glial cell macrophage (GCM) in the neuropil "digesting" a small group of non-viable spores (S). ×9 000

FIG. 31 Perithelial cell macrophage (PCM) with a non-viable spore and abundant lysosomal (LY) activity (arrows). This macrophage appears to be in the process of detaching itself from the vasculature. EC = endothelial cell; A = astrocyte; BL = basement lamina; RBC = red blood cell; VL = vascular lumen. ×9 000

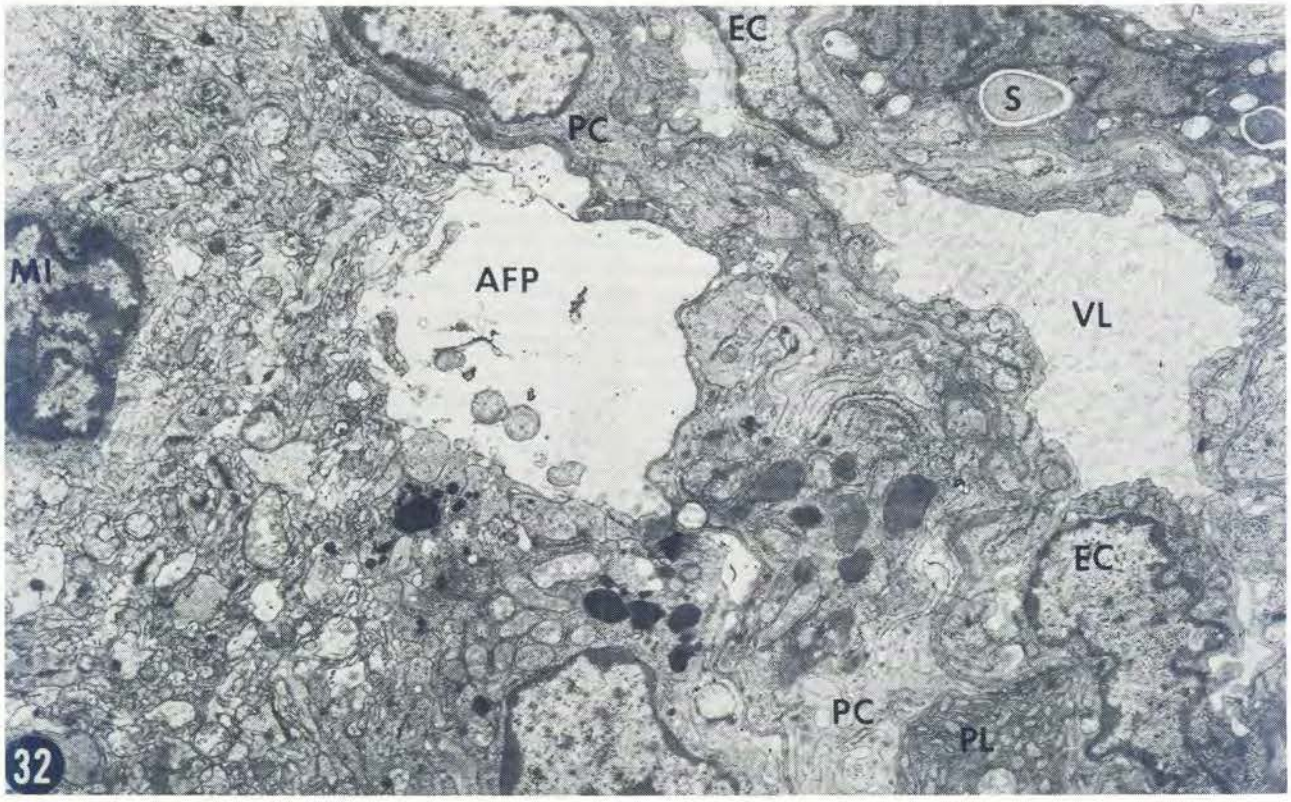
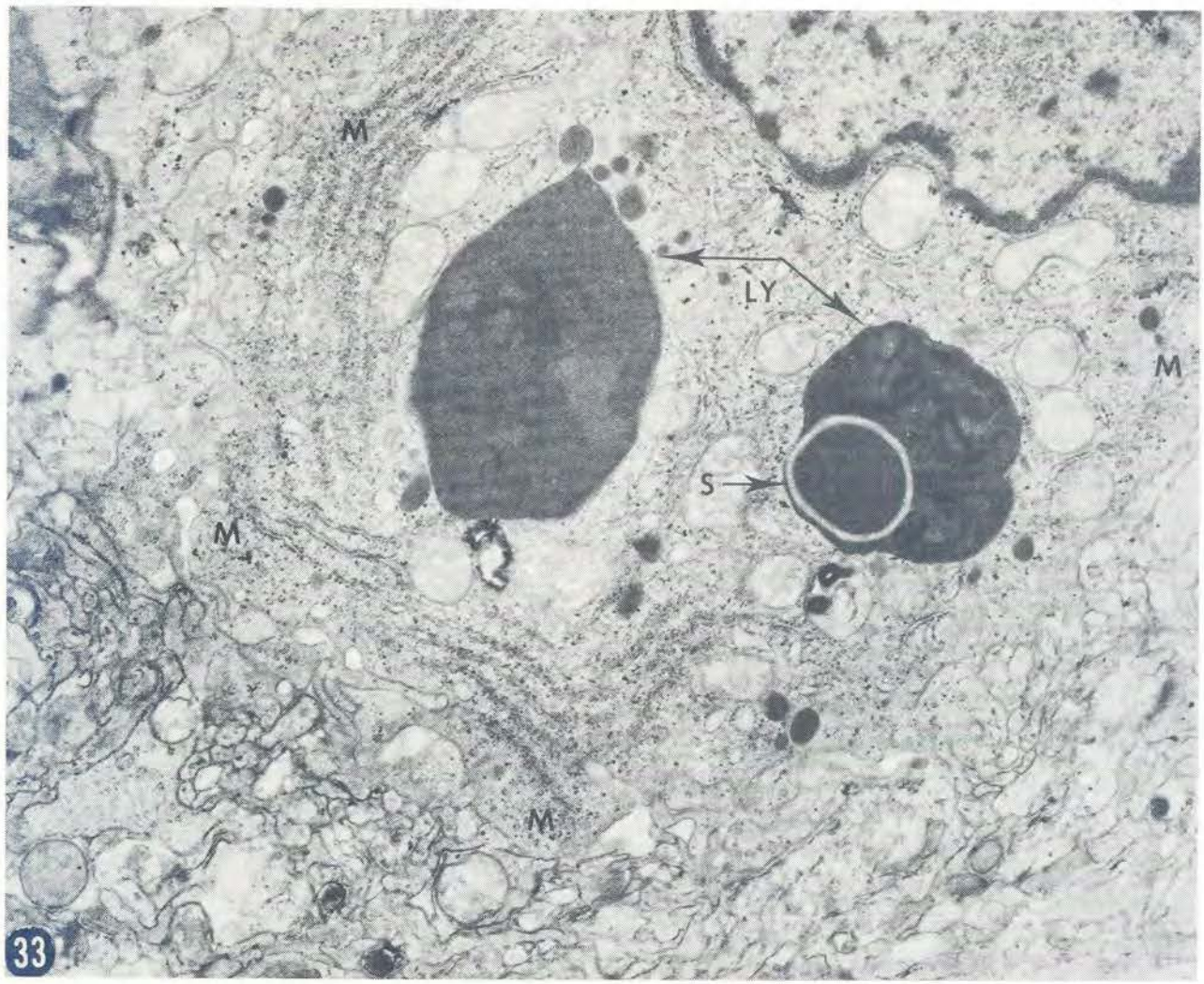


FIG. 32 A dilated astrocytic foot process (AFP) is adjacent to a parasitized vessel. A perithelial cell macrophage contains a dead spore (S). Other perithelial cells (PC), some with abundant lysosomes, also surround the vessel along with a plasma cell (PL). A microglia (MI) appears to be coming into the area from the left. EC = endothelial cell; VL = vascular lumen. ×7 250



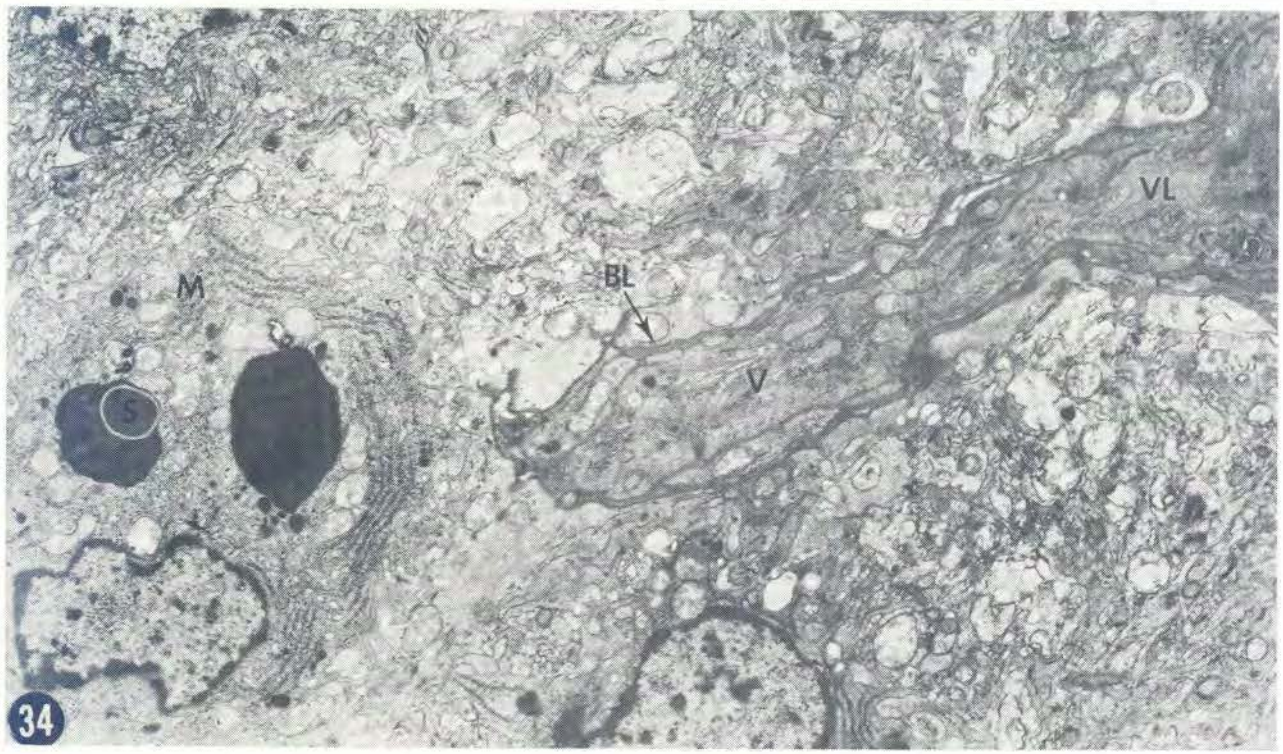
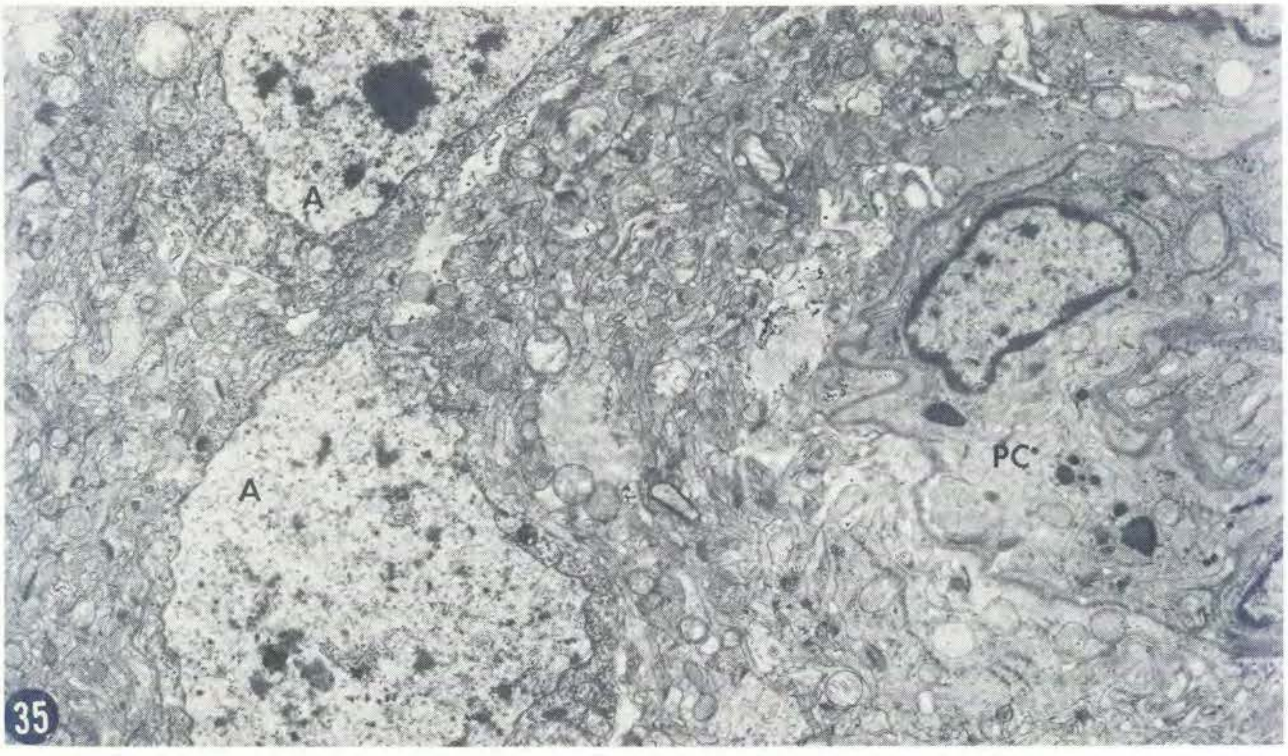


FIG. 33 Macrophage (M) with a lysosomal complex (LY) contains an almost completely "digested" spore (S). Only the remnant spore wall and its characteristic clear space (halo) identify this non-viable spore. ×17 500

FIG. 34 Macrophages (M) with non-viable spores (S) in advanced stage of degeneration were often present near healing vessels (V). VL = vessel lumen; BL = basement lamina. ×7 500



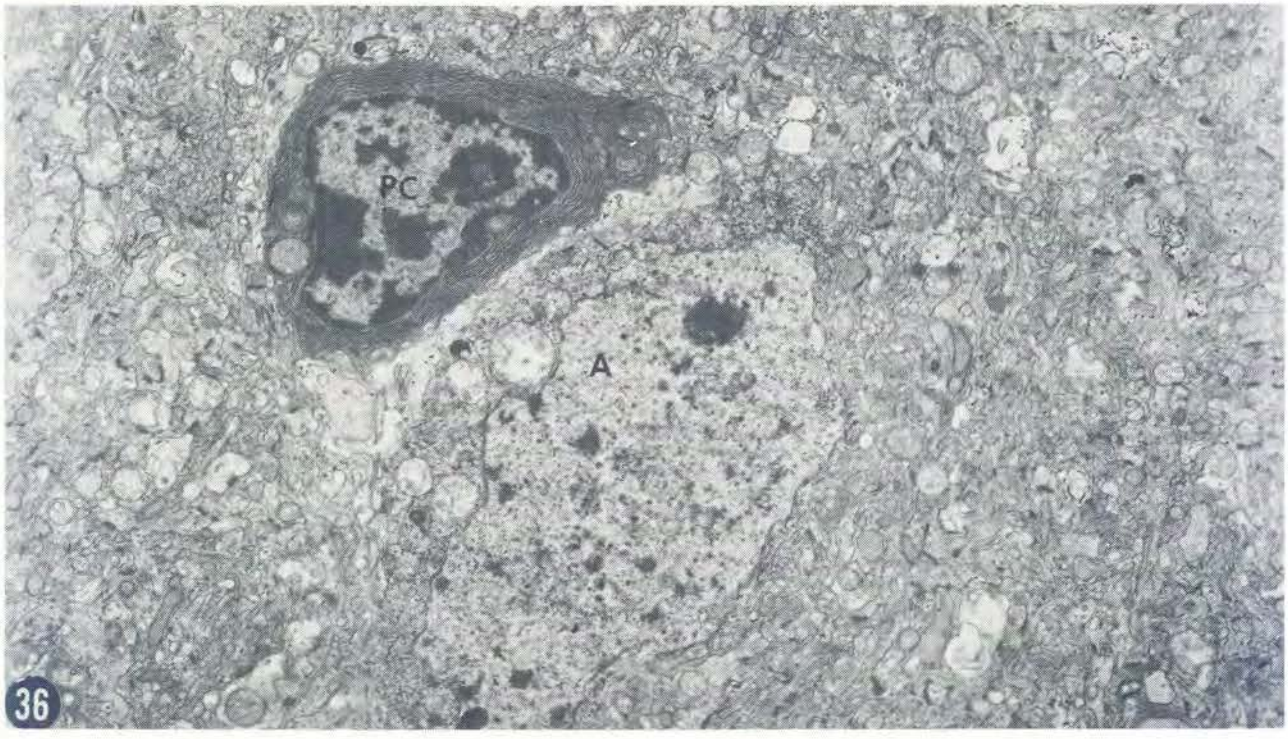


FIG. 35 Proliferating astrocytes (A) in ultrastructurally normal neuropil surround a vessel with reactive perithelial cells (PC). ×6 750

FIG. 36 Astrocytes (A) were often seen in the presence of plasmacytes (PC) which were percolating through ultrastructurally normal neuropil. ×6 750

Non-viable spores were found in different locations, the most frequent site being within perithelial cell macrophages attached to parasitized vessels (Fig. 32). They were also present within macrophages which appeared to be in the process of detaching themselves from parasitized vessels (Fig. 31). Finally, they were seen within glial cell macrophages migrating in the neuropil (Fig. 30). Spores within macrophages were usually surrounded by a lysosomal complex and almost completely digested, the clear space associated with the cell wall being the most conspicuous remaining feature (Fig. 33). These macrophages with spores in advanced stages of digestion were often seen in proximity to apparently healing vessels (Fig. 34).

Proliferating astrocytes were usually peripheral to the perithelial and epithelioid cell reaction (Fig. 35), and were often accompanied by lymphoid cells, such as plasmacytes percolating through the neuropil (Fig. 36).

Discussion

Canine encephalitozoonosis (nosematosis) has been referred to by Plowright (1952) as the encephalitis-nephritis syndrome based on the consistent finding of lesions in the brain and kidney. This general view may be too narrow since other organs are also consistently parasitized and diseased [Plowright, 1952; Plowright & Yeoman, 1952; McCully *et al.* (in press); Van Dellen & Botha, unpublished observations]. Indeed, the organ most consistently affected is the vascular system. Prior to this report vasculitis had not been emphasized as a basic lesion of canine encephalitozoonosis.

The aetiological agent's specific histochemical stain reactions, its size and shape, the presence of a polar vacuole and filament and the type of lesion produced suggested that it was a microsporidian. These characteristics differentiated the agent readily from *Toxoplasma*. The presence of a spore filament and a single nucleus throughout its reproductive life cycle, as observed ultrastructurally, not only confirmed that it was *Encephalitozoon* in accordance with recent work published by Cali (1970) and Sprague & Vernick (1971), but also differentiated this organism from *Nosema*.

Clinical signs of disease in young dogs with *Encephalitozoon* infection vary considerably and range from none (Botha & Van Dellen, unpublished observation) to severe CNS disturbance, coma and death. Poor growth, ataxia, tremors, and blindness are characteristic features of the disease in its acute and subacute forms. The 2 cases described above reflect the expected variability of clinical disease. Partial blindness was essentially the only serious clinical abnormality in Pup B, whereas Pup A revealed advanced CNS disease, not unlike that of canine distemper, and a gradual deterioration towards coma and eventual death. Clinical signs shown by Pup A at the time of necropsy resembled advanced canine distemper. Although distemper is usually the most important differential diagnosis with respect to encephalitozoonosis, it was not considered probable in the case of Pup A because it had drunk colostrum of a bitch which had been vaccinated against distemper 8 months prior to whelping. Furthermore, the pup showed clinical signs of disease during its first week of life, which is unlikely with distemper.

Gross changes in the brain have not been reported, nor were any seen in either of these cases. However, histological changes in the brain were always present, which in Pup A were mostly acute with diffuse gliosis and severe vasculitis, the latter causing extensive oedematous change. Lesions in the brain and spinal cord of Pup B consisted primarily of a subacute to chronic cellular reaction of vessels, with little oedema, developing into prominent nodular, granulomatous vasculitis.

This vasculitis in the brain is distinct in routine HE histopathologic sections and is recognizable from its pattern and specific type of cell reaction, and distribution of affected vessels. However, the lumen or other characteristics of a vessel were not always apparent. In this regard the study of 1–2 μm thick plastic sections was especially useful in that cell detail and lesion anatomy was more definitive, making it clear that the underlying pathological change was in the vascular system. Parasitized cells with viable organisms were consistently endothelial cells, and reactive perithelial cells appeared to be the major contributor to perivascular cellular inflammation. Lymphoid cells infiltrated these areas, and glial cells were reactive in the neuropil surrounding the involved vessel.

Ultrastructural study of the brain confirms the above histological observation in greater detail. It is remarkable that a macrophage process may surround the entire granulomatous reaction which often consists of 3 or more concentric layers of epithelioid cells. Perivascular macrophages and epithelioid cells appear to be reactive, transformed perithelial cells which often contain degenerated parasites.

What readily became apparent from the ultrastructural study of these 2 cases was that viable organisms in the brain never appeared anywhere other than within endothelial cells. When spores were seen elsewhere, they were most often within a perithelial cell macrophage and rarely within a glial macrophage (which may in fact be detached perithelial cell macrophages), apparently moving within the neuropil. Correlation of histological and ultrastructural features of spores within macrophages confirmed that these spores are non-viable. Their round to oval, internal, homogeneous mass contained no organellae, suggesting that they were being subjected to lytic processes. Indeed, lysosomes were usually in close proximity to these spores. Histologically, the material of non-viable spores which stains positive with PAS and GMS is the round to oval internal homogeneous mass seen ultrastructurally. The translucent space which surrounds the black (GMS) or reddish-pink (PAS) mass is a characteristic of these non-viable spores, even when they are in advanced stages of degeneration. It appears as a "halo" and represents an apparent artifactual space between the parasite's degenerated cytoplasmic membrane and remnant spore wall.

The electron translucent space of non-viable spores, also present, though not as wide, when they are viable and within endothelial cells, seems to develop simultaneously with increasing electron density and maturation of the spore wall. A mature spore wall, being dense and undoubtedly hard, is probably less subject to shrinkage than the internal, softer components of the parasite within this rigid shell. Internal components of the spore would therefore be expected to shrink by a greater percentage than the spore wall, thus creating the artifactual translucent space.

A group of organisms, apparently free within the neuropil, as seen with routine HE 6 μm histologic sections, is in fact within an endothelial cell when studied with 1–2 μm plastic sections. Electron microscopy confirmed this observation and established that

the organisms are within the endothelial cell's distended, cytoplasmic sporogony vacuole and not membranebound. When the vacuole is at its maximum expansion the thin remaining strand of cytoplasm ruptures easily, thereby releasing spores into the vascular lumen.

The vegetative stage within host cell cytoplasm is presumed to arise by multiple binary fission. However, no evidence of division at this stage was observed, and therefore the genesis of this stage, and perhaps of an earlier stage, is not entirely clear. Further study of the organism at the early stage of its reproductive life cycle is needed to clarify the exact mode of proliferation.

Phagocytic macrophages containing non-viable spores when encountered in the neuropil are believed to be cells which have migrated from the vasculature after having phagocytized organisms. Their exact identity could not be determined, but they most probably originate from either the perithelial or glial cell. We are inclined to believe that they are detached perithelial cells, since these cells with phagocytized spores have been observed in a semi-detached position, whereas spores were not unequivocally identified within glial processes in association with vessels.

Not all spores in the sporogony vacuole are acid-fast or gram-positive, and only some polarize light. A study of them in plastic HE sections revealed that some have a combined basophilic/eosinophilic appearance, while others are entirely eosinophilic or basophilic. With the aid of electron microscopy, we arrived at the conclusion that variable stain reactions indicate different stages of spore maturation, especially of the spore wall, and electron micrographs show that the spore wall changes in thickness and electron density as the spore matures. Corresponding biochemical changes undoubtedly occur to account for the different histochemical reactions.

It appears that acute cases of encephalitozoonosis will most probably have a larger number of viable than non-viable spores, as in Pup A, and therefore the Gram stain is the best choice for diagnostic identification of the spore. However, when dealing with subacute to chronic suspect cases of the disease, as in Pup B, the PAS and GMS stains are the best choice for diagnostic work. In the latter situation, the non-viable spore with its characteristic "halo" will be quickly identified within macrophages of perivascular inflammation.

Fibrinoid necrosis accompanying segmental, nodular vasculitis was rarely observed in either of these cases. This is in contrast to other reported cases of this disease in dogs and the blue fox in which this pathological change was a remarkable finding and leads to the suggestion that this vascular lesion has features in common with periarteritis nodosa. A state of hypersensitivity was suggested as the basis for this lesion in canine encephalitozoonosis. The morphological similarity of this lesion to periarteritis nodosa is obvious, but its immunological basis will require clarification with more research. If the vascular lesion in the canine is indeed a true periarteritis nodosa, we would have expected to find a significant number of these lesions in Pup B with subacute encephalitozoonosis where a state of hypersensitivity should have been established. We are inclined to believe, however, that necrotic vasculitis with encephalitozoonosis usually results from a vascular ischemia. This may result from occlusive parasitaemia of endothelial cells at 2 different, though close, points in 1 vessel. Also, focal parasitaemia of endothelial cells of the vasa vasorum may cause occlusion of these very small vessels. Even so, different lesions in different animals undoubtedly reflect a multiplicity of factors, including variance in the host's immunological defence status and perhaps variable pathogenicity potential of different species or strains of parasite. Further research is necessary to clarify the pathogenesis of the necrotic vascular lesion.

Although we have occasionally seen intraneural parasitism in other cases, neither light nor electron microscopy revealed parasites within neurons of these 2 cases. From this study it in fact became clear that apparent intraneural organisms are probable within a juxtaposition capillary endothelial cell. Such an endothelial cell, when filled and distended with organisms, may push tightly against a contiguous neuronal cytoplasm. This may displace the neuron's cytoplasm and make its nucleus appear as part of a compact group of parasites actually within an endothelial cell whose own nucleus is obscured. The limitations of a relatively thick histological section (6 microns) contribute to the illusion of intraneural parasitism. Furthermore, if intraneural parasitism did indeed play a significant part in the pathogenesis of this disease, one would expect to find frequent evidence of satellitosis and neuronophagia, but this manifestation of nerve cell injury was not observed in relation to intraneural parasitism. Injury to nerves only occurred when they were caught up in the inflammatory reaction directed against vascular parasitism. Intraneural parasitism may occur, but it is undoubtedly uncommon with encephalitozoonosis and not important in connection with the pathogenesis.

Parasitological and pathological changes affecting cerebral vessels, as observed in these 2 natural cases, reflect the basic morphological features of canine encephalitozoonosis affecting the brain, and we suggest that they represent the underlying factors in the pathogenesis of this disease.

Acknowledgements

We wish to thank the technical staff of the Pathology Section at Onderstepoort and the Department of Pathology, Veterinary Faculty, University of Pretoria, for the preparation of histological sections. The dedicated assistance given by Miss Hannelie de Lange in the excellent preparation of sections for the electron microscope is greatly appreciated. We also acknowledge with thanks the advice, encouragement, and help generously given by Dr J. G. Pienaar in the preparation of this manuscript.

References

BASSON, P. A., McCULLY, R. M. & WARNES, W. E. J., 1966. Nosematosis: Report of a canine case in the Republic of South Africa. *Journal of the South African Veterinary Medical Association,* 37, 3–9.

CALI, A., 1970. Morphogenesis in the genus *Nosema. Proceedings of the 4th International Colloquium of Insect Pathology,* pp. 431–438.

CHANG, S. C., 1972. Hematoxylin-eosin staining of plastic-embedded tissue sections. *Archives of Pathology,* 93, 344–351.

HAYAT, M. A., 1970. *Principles and Techniques of Electron Microscopy. Biological Applications. Vol I.* New York: N.Y., Van Nostrand Reinhold Co.

INNES, J. R. M., ZEMAN, W., FRENKEL, J. K. & BORNER, G., 1962. Occult endemic encephalitozoonosis of the central nervous system of mice (Swiss-Bagg-O'Grady strain). *Journal of Neuropathology and Experimental Neurology,* 21, 519–533.

KANTOROWICZ, R. & LEWY, F. M., 1923. Neue parasitologische und pathologisch-anatomische Befunde der nervösen Staupe der Hunde. *Archiv für Wissenschaftliche u. praktische Tierheilkunde,* 49, 137–157.

KAY, H. D., 1965. *Techniques for Electron Microscopy*. 2nd ed. Oxford, Blockwell Scientific Publications.

KOLLER, L. D., 1969. Spontaneous *Nosema cuniculi* infection in laboratory rabbits. *Journal of the American Veterinary Medical Association*, 155, 1108–1114.

LEVADITI, C., NICOLAU, S. & SCHOEN, R., 1923. L'etiologie de l'encephalite. *Comptes Rendus des Seances de L'academie des Science*, 177, 985–988.

LUNA, L., 1968. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. Third Edition.

MALHERBE, H. & MUNDAY, V., 1958. *Encephalitozoon cuniculi* infection of laboratory rabbits and mice in South Africa. *Journal of the South African Veterinary Medical Association*, 29, 241–246.

MARGILETH, A. M., STRANO, A. J., CHANDRA, R., NEAFIE, R., BLUM, M. & McCULLY, R. M., 1973. Disseminated nosematosis in an immunologically comprised infant. *Archives of Pathology*, 95, 145–150.

NORDSTOGA, K., 1972. Nosematosis in blue foxes. *Norwegian Veterinary Medicine*, 24, 21–24.

OLIVER, J., 1922. Spontaneous chronic meningo-encephalitis of rabbits. *Journal of Infectious Diseases*, 30, 91–94.

PEASE, D. C., 1964. *Histological Techniques for Electron Microscopy*. 2 ed., New York, N.Y., Academic Press.

PERRIN, T. L., 1943. Spontaneous and experimental *Encephalitozoon* infection in laboratory animals. *Archives of Pathology*, 36, 559–567.

PLOWRIGHT, W., 1952. An encephalitis-nephritis syndrome in the dog probably due to congenital *Encephalitozoon* infection. *Journal of Comparative Pathology*, 62, 83–92.

PLOWRIGHT, W. & YEOMAN, G., 1952. Probable *Encephalitozoon* infection of the dog. *The Veterinary Record*, 62, 381–383.

SPRAGUE, V. & VERNICK, S. H., 1971. The ultrastructure of *Encephalitozoon cuniculi* (Microsporidia, Nosematidae) and its taxonomic significance. *The Journal of Protozoology*, 18, 560–569.

VAN RENSBURG, I. B. J. & DU PLESSIS, J. L., 1971. Nosematosis in a cat: A case report. *Journal of the South African Veterinary Medical Association*, 42, 327–331.

WRIGHT, J. H. & CRAIGHEAD, E. M., 1922. Infectious motor paralysis in young rabbits. *Journal of Experimental Medicine*, 36, 135–140.

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