Neuropathology of Spiroplasma Infection in the Rat Brain

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This study was designed to demonstrate the neuropathology of persistent spiroplasma infection in the rat brain. GT-48 spiroplasmas were inoculated intracranially into a series of suckling Sprague–Dawley rats. Their brains were evaluated at specific time intervals by microbiologic assay and by morphologic studies including histology, electron microscopy, and immunocytochemistry. The spiroplasmas were observed in the tissues by electron microscopy at peak infection 14 days after intracranial inoculation. At that time they were seen in vacuoles and neuronal processes within the neuropil as filamentous or bleblike forms. A single tight spiral was identified that closely resembled the spiroplasmalike inclusions previously reported in Creutzfeldt–Jakob disease. The spiroplasmas were shown to spread rapidly throughout the brain tissues presumably by intraneuronal transport. In specimens examined at 25 days after intracranial inoculation and beyond, organisms were localized to gray matter without inflammatory response. The spiroplasmas could not be identified by electron microscopy in the rat brain tissue at late stages of infection. This study has shown an unusual adaptation of spiroplasma infection to the mammalian host brain tissues. (Am J Pathol 1984, 114: 496–514)

THE SUCKLING MOUSE cataract agent (SMCA) and a second related strain (GT-48) have been identified as members of a group of helical mycoplasmas (*Spiroplasma mirum*).[1–3] Both of these wall-free prokaryotes, which were initially isolated from rabbit ticks,[4,5] can induce a variety of pathologic changes (including cataracts) in young rodents.[4–6] They are particularly interesting from a neuropathologic standpoint, because they have been shown to produce a persistent brain infection in 1–2-day-old suckling rats or mice.[5,7–9] Limited ultrastructural examination of brain tissue has failed to localize the microbe, even in tissues where as many as 10^9 organisms/g have been recovered in culture. In this report, the GT-48 strain of *S mirum* was employed in an experimental infection of rat brain tissues examined at periodic intervals by immunocytochemistry and transmission electron microscopy.

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Materials and Methods

Preparation of Inoculum

The GT-48 strain of *S mirum* was grown in SP-4 broth[2] culture for 5 days at 30 C. The stock broth culture was serially diluted in 10-fold steps with sterile culture medium to provide challenge dilutions of 10^{-5} and 10^{-6}, representing approximately 300 to 30 organisms, respectively, per 0.03 ml of inoculum. Following challenge, the vials containing serial dilutions of the culture were incubated at 30 C for 21 days. The number of organisms in the stock culture, and consequently administered to animals, was estimated from the presence of helical forms in individual tubes of the dilution series by darkfield microscopy. The stock culture usually contained 10^9 organisms/ml.

Experimental Animals

One-day-old Sprague-Dawley rats (this is a conventional colony with no barrier system obtained from Taconic Farms, Germantown, New York) were used in the study. Groups of neonatal rats were inoculated intracranially with the 0.03 ml of diluted GT-48 spiroplasma broth culture. Inoculum was injected into the right hemisphere of the brain in each rat. Additional groups of rats (controls) were similarly inoculated with 0.03 ml doses of sterile culture medium.

Tissue Collection

Groups of infected and control rats were killed at selected time intervals between 7 and 60 days following intracranial inoculation. Two groups of infected and control animals were examined at each of the time points. Brains from one group of control and infected rats were removed aseptically and cultured for spiroplasmas by the following technique: the brain was placed in a sterile plastic dish and minced with the use of scissors. The tissue was then added to 5 ml of SP-4 broth (approximately a 1:10 dilution). Serial 10-fold dilutions of the brain suspension were made in SP-4 broth. All tubes were then incubated for 21 days at 30 C. At the end of this period, the tubes were examined for the presence of helical spiroplasmas, with the use of darkfield microscopy. The number of spiroplasmas in brain suspensions was calculated from the highest 10-fold dilution showing helixes. The second group of control and infected rats was anesthetized and cardiac-perfused with 2% glutaraldehyde/2% formaldehyde in phosphate-buffered saline (pH 7.4). After perfusion, the brains were removed from the rats and prepared for histopathologic, immunocytochemical, and ultrastructural examination. In this study the results obtained at 14 and 25 days after intracranial inoculation are reported.

Histopathologic and Electron-Microscopic Preparation

Paraffin-embedded brain tissues from control and infected rats were sectioned at 5 μ, stained with hematoxylin and eosin (H&E), and used for routine histopathologic examination. Additional selected samples of brain from control and infected rats were embedded in Epon and serially sectioned for both transmission electronmicroscopy and immunocytochemistry. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 100 transmission electron microscope.

Immunocytochemical Preparation

Paraffin and Epon-embedded brain tissues from control and infected rats were studied using the unlabeled antibody peroxidase-antiperoxidase (PAP) method.[10] Plastic was removed from the Epon-embedded tissue prior to the immunocytochemical studies by treatments of the sections with a diluted preparation of sodium ethoxide.[11]

Immunochemistry was performed as follows. Endogenous peroxidase activity was abolished by emersion of the tissue sections into absolute methanol containing 3% hydrogen peroxide for 30 minutes. Sections were then passed through graded alcohol solutions to water, then blotted and treated with undiluted goat serum for 30 minutes for reduction of nonspecific immunostaining. The sections were shaken for removal of the goat serum, and a specific antiserum to GT-48 spiroplasmas was prepared in rabbits (diluted 1:240 in phosphate-buffered saline [PBS] containing 2.5% goat serum) was applied to the sections for 1 hour. The treated sections were washed three times with Tris buffer (0.5 M at pH 7.6) with a volume of 100 ml and 5 minutes per change. Goat antirabbit IgG (1:64 dilution in PBS plus 2.5% goat serum) was added to the sections for 30 minutes. The sections were washed with Tris buffer as before and treated with rabbit PAP (1:120 dilution of PAP preparation in Tris buffer plus 2.5% goat serum) for 30 minutes. The sections were washed again in Tris buffer as above and placed in a filtered, freshly prepared solution of 3,3'-diaminobenzidine tetrahydrochloride (0.05% in 0.05 M Tris buffer, pH 7.6, containing 0.3% hydrogen peroxide) for 15 minutes. Finally, the sections were washed in tap water, dehydrated, and mounted in Coverbond (Fisher Scientific).

Table 1—Numbers of GT-48 Spiroplasmas Recovered From Rat Brains at Various Intervals Following Intracerebral Challenge

Days after inoculation	Number of organisms in inoculum	Number of rats that underwent necropsy	Number of spiroplasmas recovered from individual brain suspensions
7	300	2	10^6, 10^6
	30	2	10^6, negative
	Control	1	Negative
14	300	2	10^9, 10^7
	30	2	10^9, 10^9
	Control	1	Negative
25	300	1	10^4
	30	2	10^3, negative
	Control	1	Negative
47	300	ND*	—
	30	2	10^3, 10^4
	Control	1	Negative
60	300	ND	—
	30	2	10^2, 10^3
	Control	1	Negative

* Not done; insufficient survivors were available.

Bioassay titers of infected whole rat brains show that GT-48 spiroplasma organisms grow to high titers in rat brain and produce persistent infection.

Sections of brain from infected rats were treated with preimmune rabbit serum (1:240 dilution) in place of the specific GT-48 spiroplasma antiserum and used as controls for immunochemistry. In addition similar sections were treated with the GT-48 antiserum preabsorbed with GT-48 organisms. Sections from uninfected rat brain were also examined using specific GT-48 antiserum, preimmune rabbit serum, and absorbed GT-48 antiserum. To reduce nonspecific staining during the PAP procedure, the diluted (1:240) GT-48 antiserum and preimmune serum were absorbed with 50 mg of acetone-dried normal rat brain per 5 ml of diluted sera for 1 hour at 4 C, followed by filtration.

The GT-48 antiserum was absorbed with antigen for confirmation of the specificity of the immunostaining observed in the infected brain tissues by the following means. A pellet of GT-48 organisms (grown in 1 liter of SP-4 broth) was mixed with diluted GT-48 anti-serum (1:240) and allowed to stand for 1 hour at 4 C. The GT-48 organisms were then removed by ultracentrifugation (33,000 *g* for 30 minutes). The

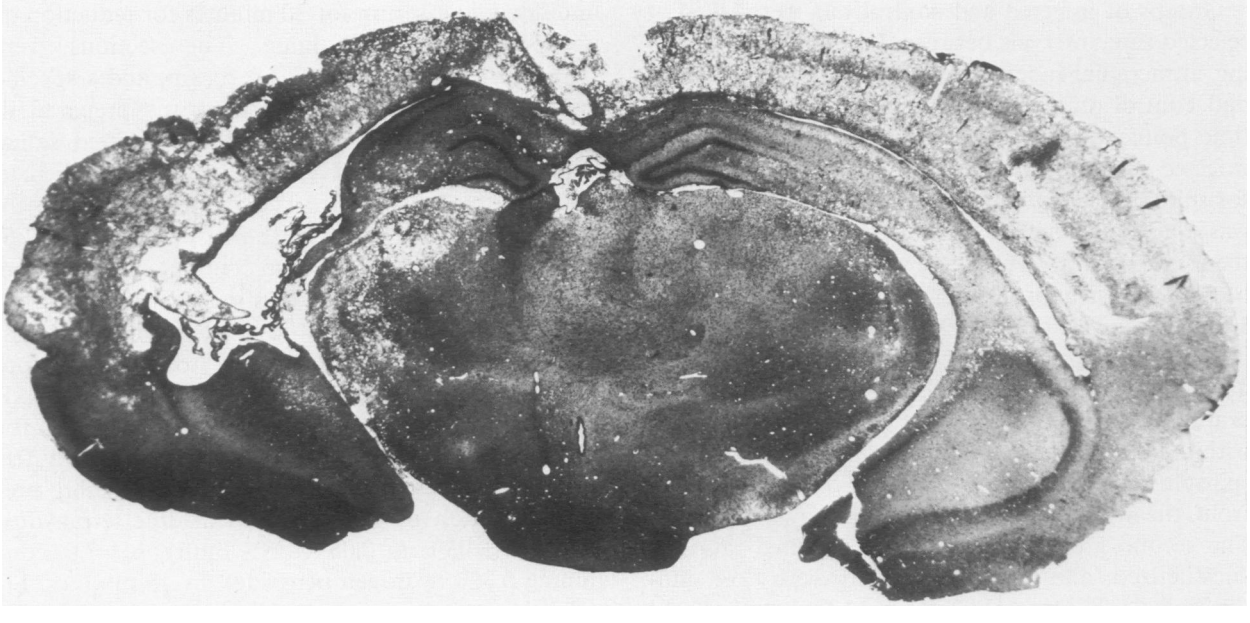
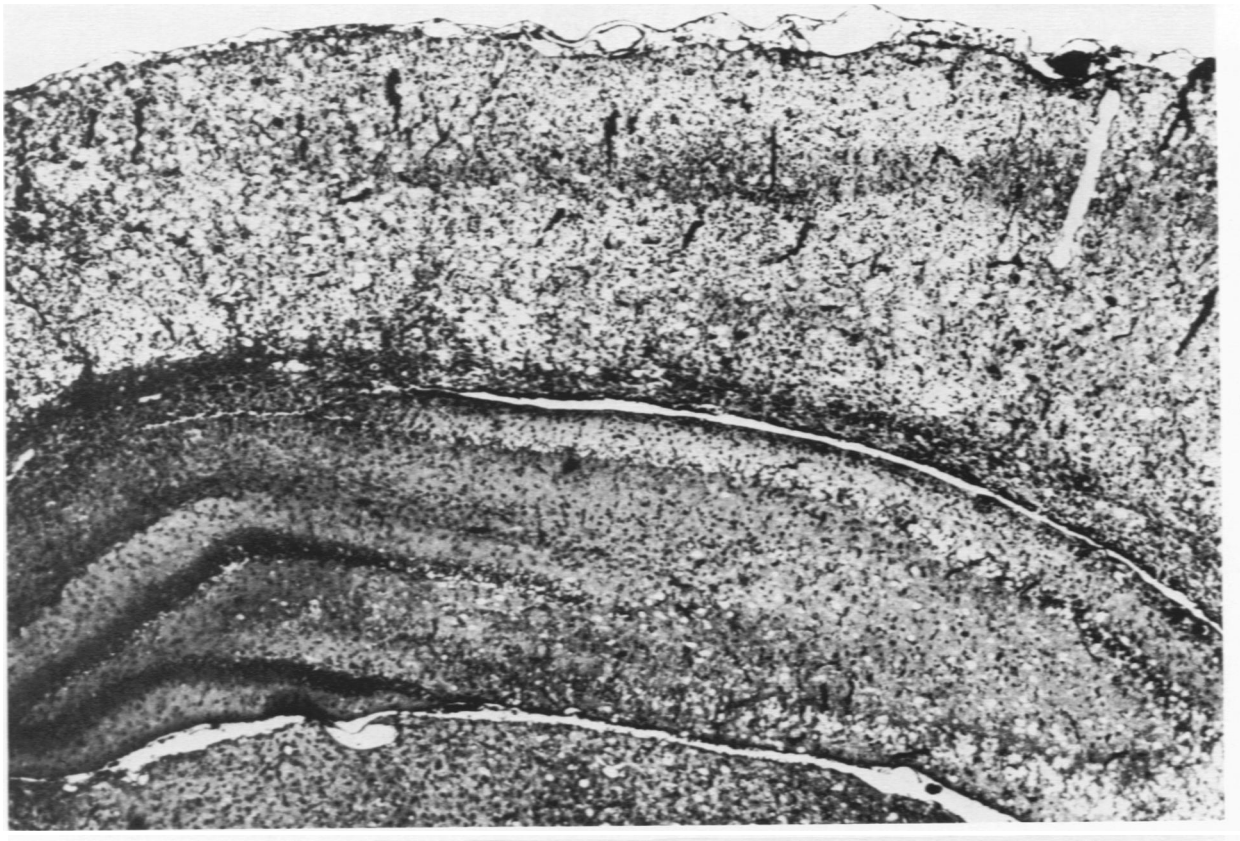


Figure 1—Coronal section of rat brain examined at 15 days after intracranial inoculation of GT-48 spiroplasmas shows microcystic encephalopathy involving the superior aspects of the cerebral hemispheres with extension into the brain stem. Bilateral vacuolar degeneration of the pyramidal neurons of the hippocampi is evident. (H&E, ×50)



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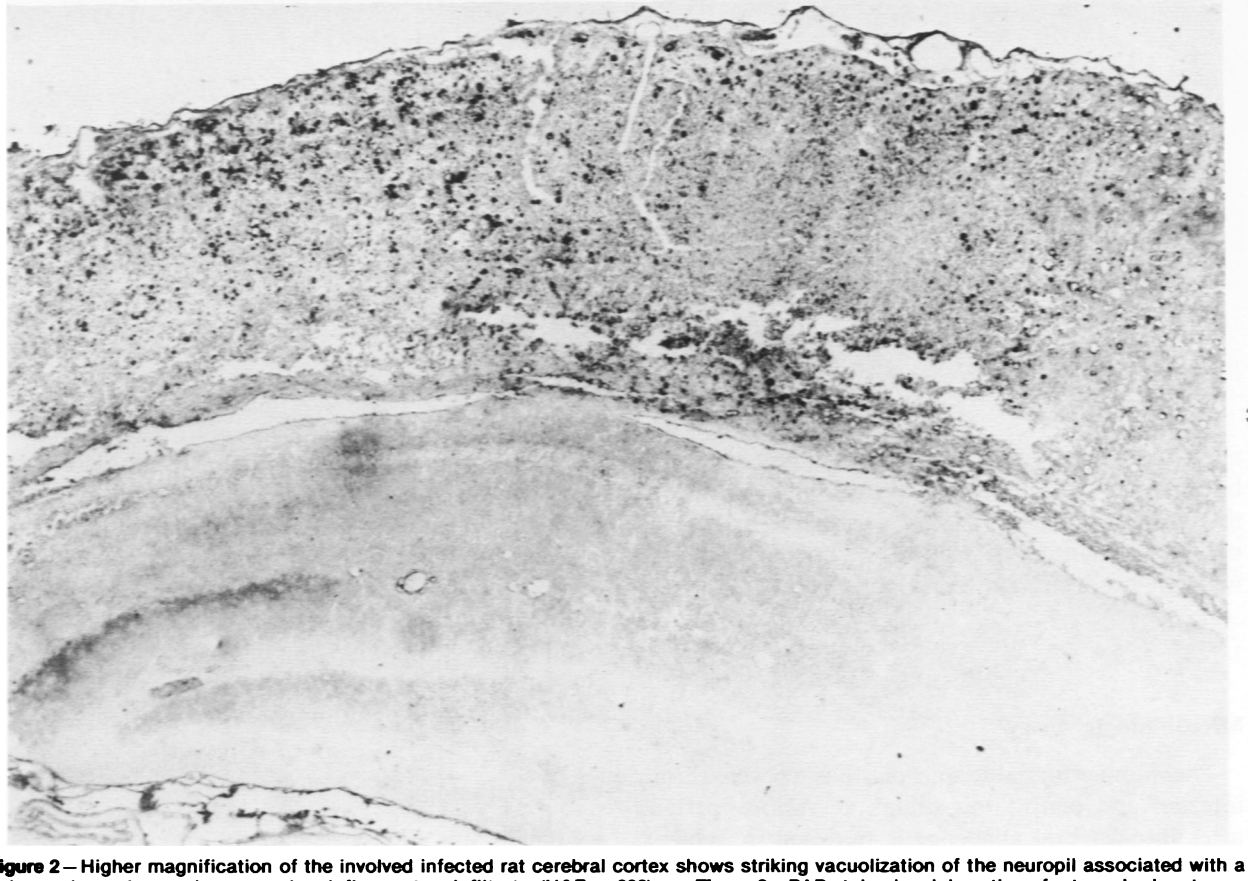

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Figure 2 – Higher magnification of the involved infected rat cerebral cortex shows striking vacuolization of the neuropil associated with a polymorphonuclear and mononuclear inflammatory infiltrate. (H&E, × 200) **Figure 3** – PAP-stained serial section of rat cerebral cortex as represented in Figure 2 after treatment with specific GT-48 spiroplasma antiserum shows immunostained bodies in the involved cortex and hippocampus. (× 200)

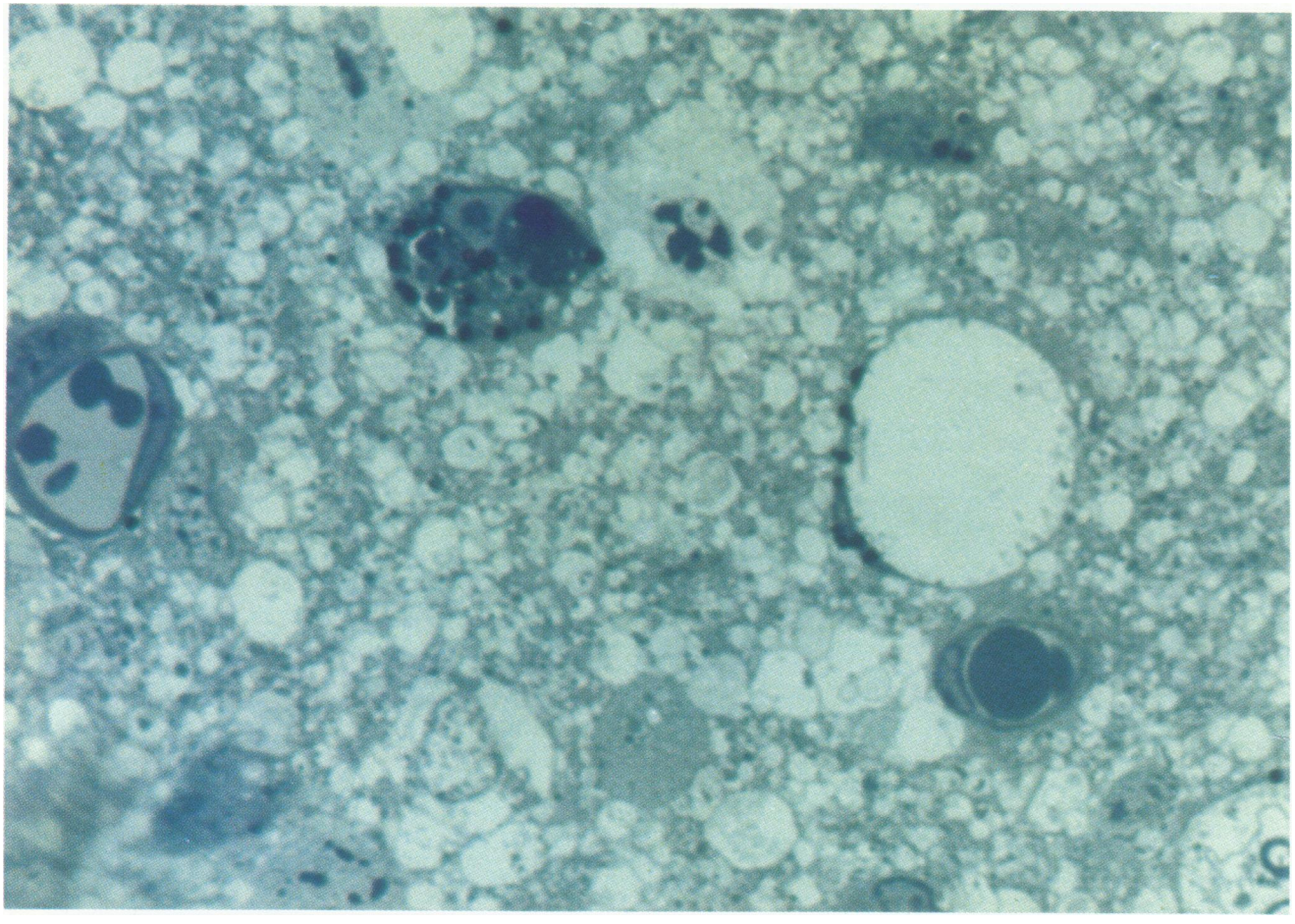


Figure 4 — Toluidine-blue-stained semithin Epon section of rat cerebral cortex 14 days after inoculation showing vacuoles within the neuropil. (×650) **Figure 5** — PAP-stained serial semithin section of rat cerebral cortex as represented in Figure 4 treated with sodium ethoxide and preabsorbed GT-48 antiserum shows absence of immunostaining. (×650) **Figure 6** — PAP-stained serial semithin section of rat cerebral cortex as represented in Figure 4 treated with sodium ethoxide and specific GT-48 antiserum shows spiroplasma antigen in vacuoles and degenerating cells within the neuropil. (×650)

effectiveness of the specific absorption was confirmed by a reduction in the metabolism inhibition titer of the serum from 1:1280 to <1:16, as well as by the lack of immunostaining of sections of infected rat brain with the absorbed antiserum.

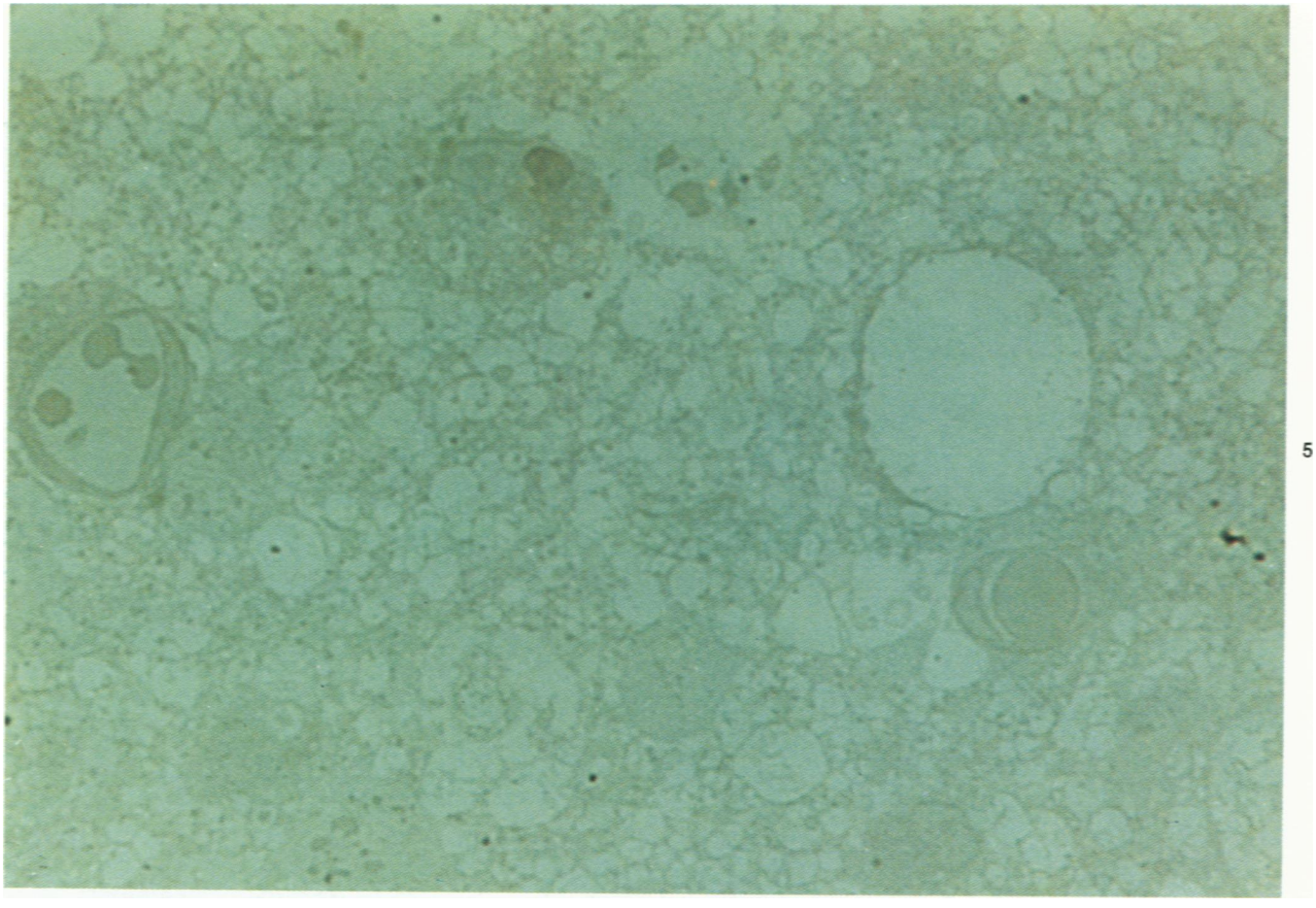
Results

Microbiologic Assay

The number of viable spiroplasmas recovered from infected and control rat brains at various periods after intracerebral challenge is recorded in Table 1. Rats that underwent necropsy 7 days after challenge usually contained 10^6 organisms in the brain, regardless of the challenge dose administered. However, in one rat, no organisms were grown from brain tissue. At Day 14, the 4 rat brains examined contained between 10^7 and 10^9 viable *Spiroplasma* organisms. Infectious titers of brain tissue declined thereafter, although the rats that underwent necropsy at 60 days still possessed viable organisms. No *Spiroplasma* organisms were recovered from brains of rats inoculated with sterile culture medium.

Histopathology

The brains of rats examined histologically 14 days after intracranial inoculation of GT-48 *Spiroplasma* organisms revealed a microcystic encephalitis. This



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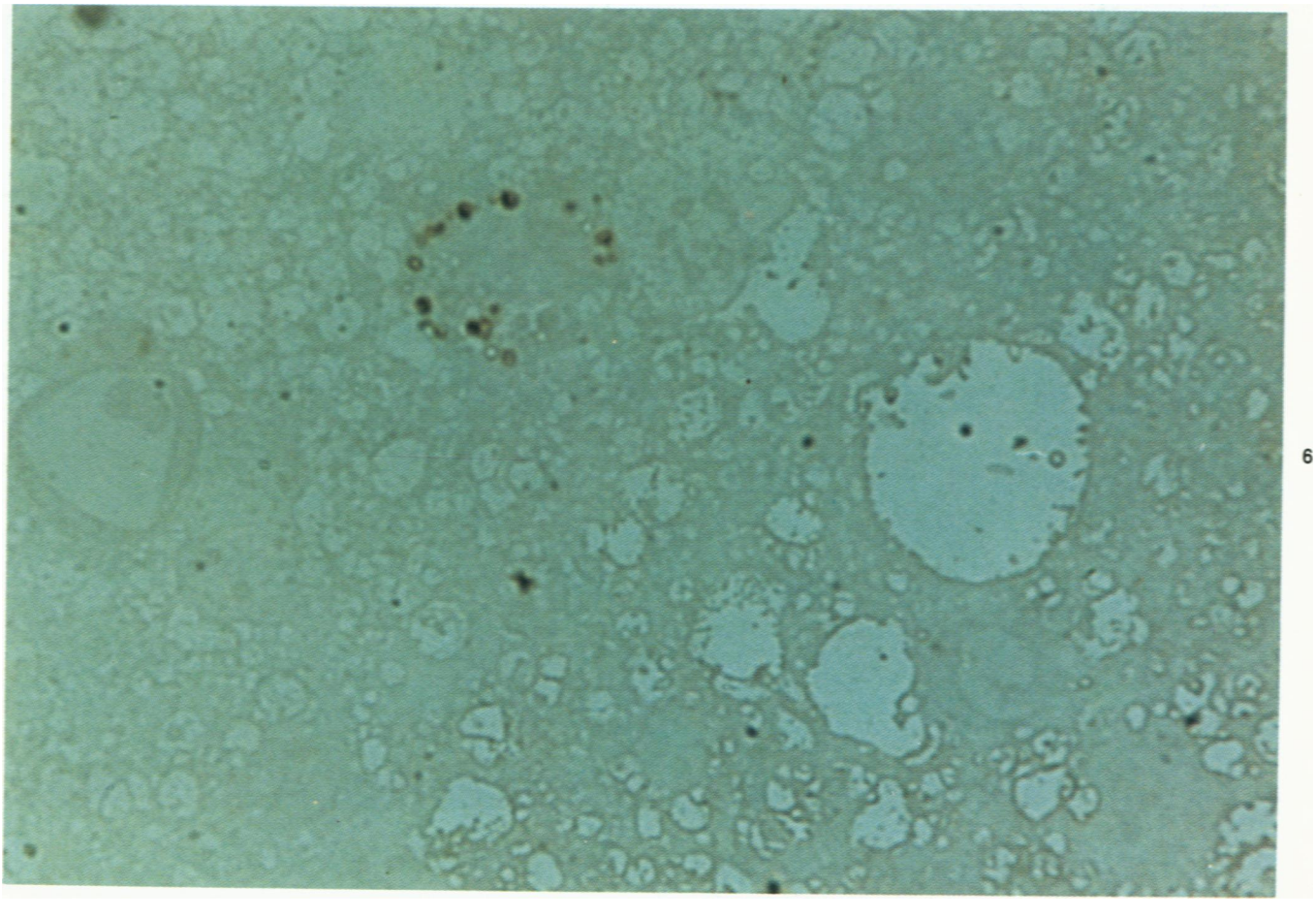

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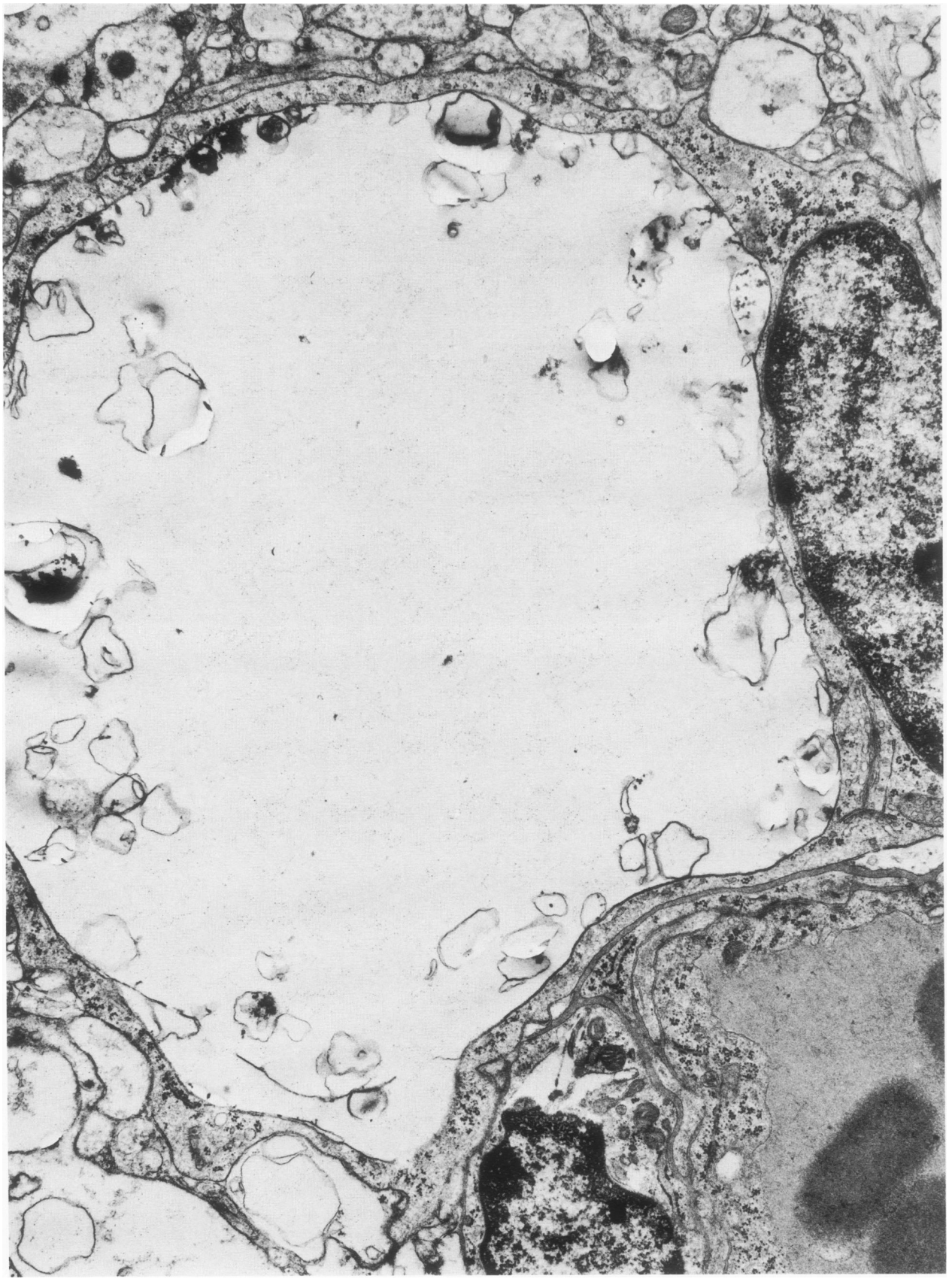


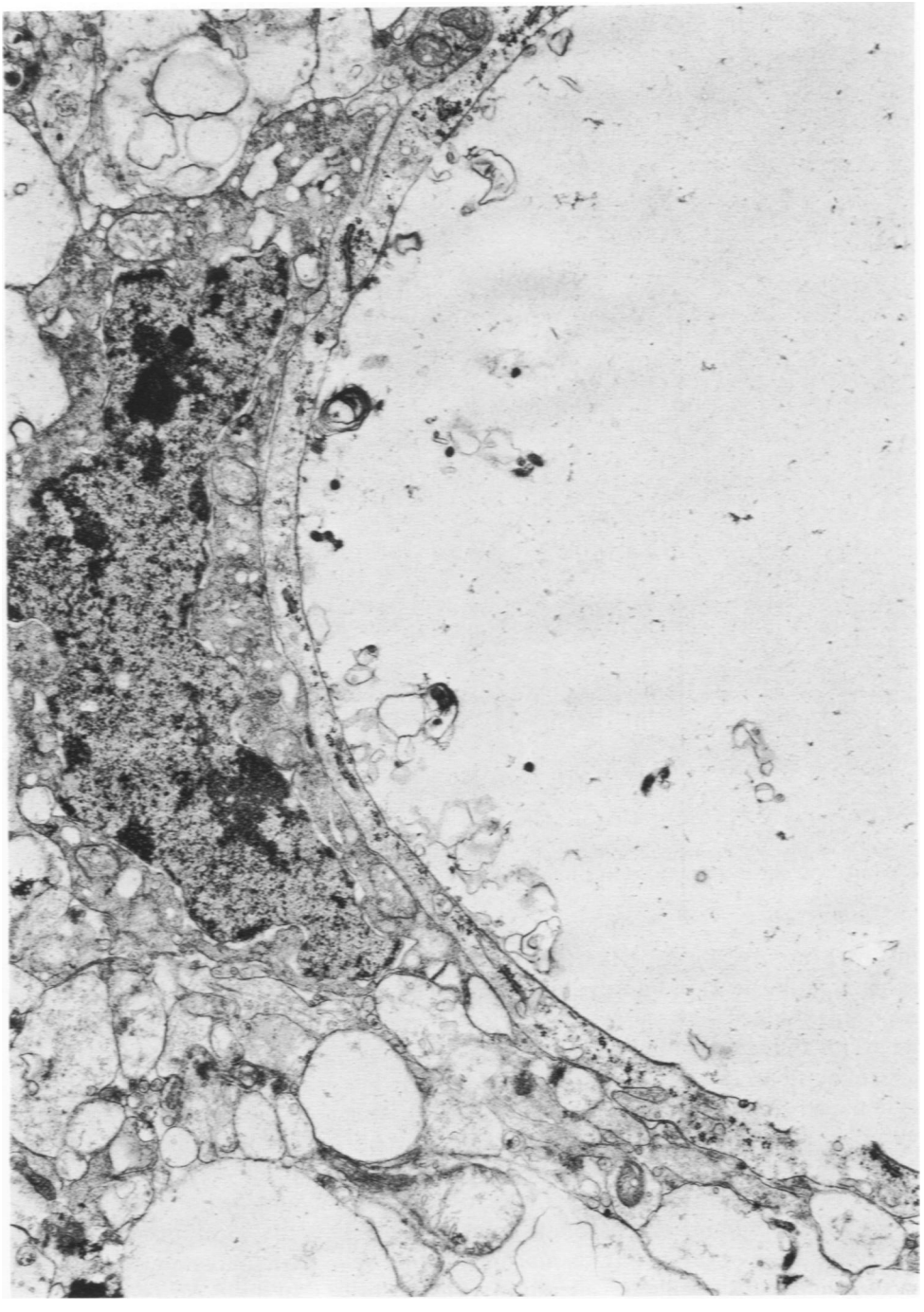


Figure 8 – Low-power electron micrograph of a microcystic area from spiroplasma-infected rat cerebral cortex shows membranous bleb forms aligned along the periphery of another vacuole corresponding to immunostained bodies seen by PAP. Notice the membrane defect in the wall of the vacuole, which may represent the base of bleblike membranous formation. (×14,500)

Figure 7 – Low-magnification electron micrograph of 14-day-old rat infected with GT-48 *Spiroplasma* representing a thin section adjacent to the thick sections seen in Figures 4–6 (method of Trapp[11]). Notice the membrane profiles aligned along the wall of a large vacuole, which correspond to the spiroplasma antigen demonstrated within the vacuole in Figure 6. (×13,500)

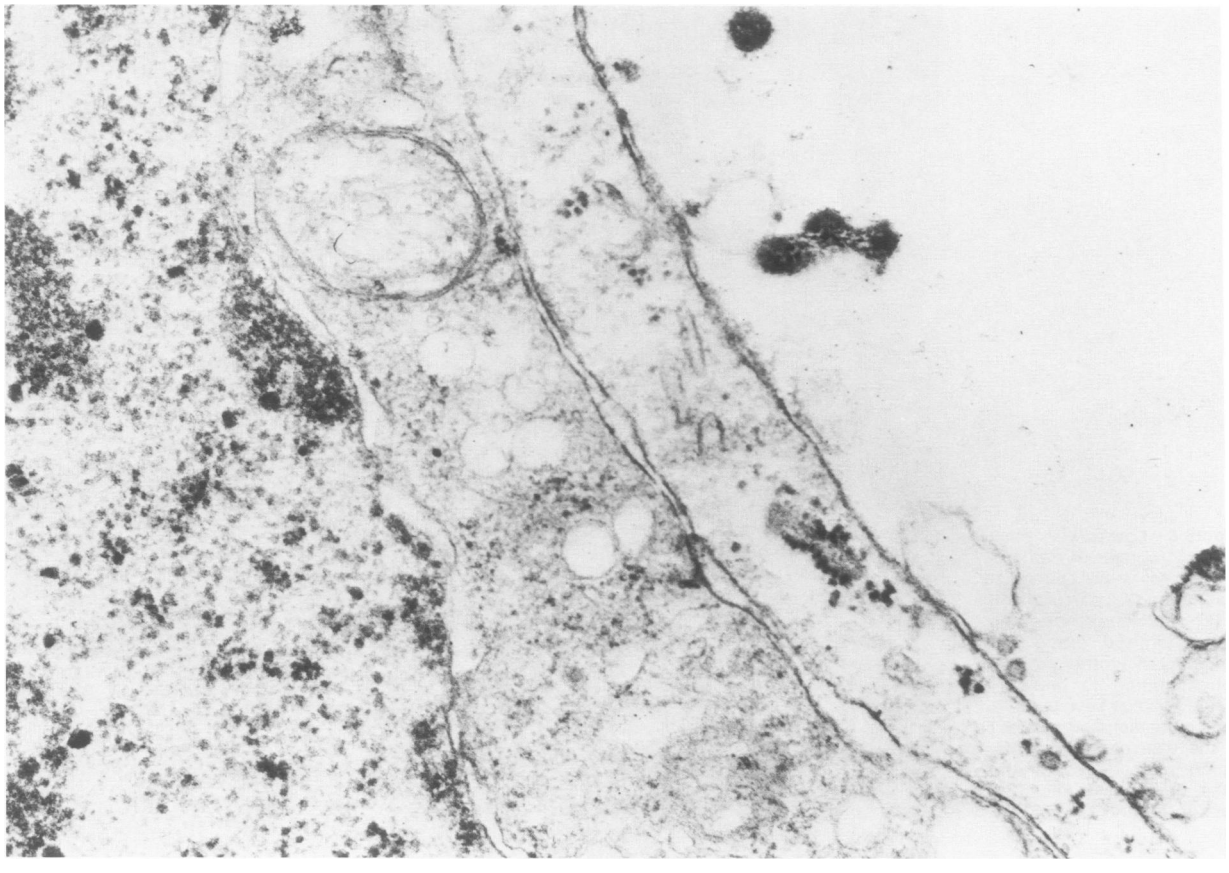


Figure 9 — A filamentous form of *Spiroplasma* (recognizable by comparison with a centrifuged pellet of *in vitro* culture in Figures 12 and 13) is seen along with membranous blebs within the vacuole represented in Figure 8. (×30,000)

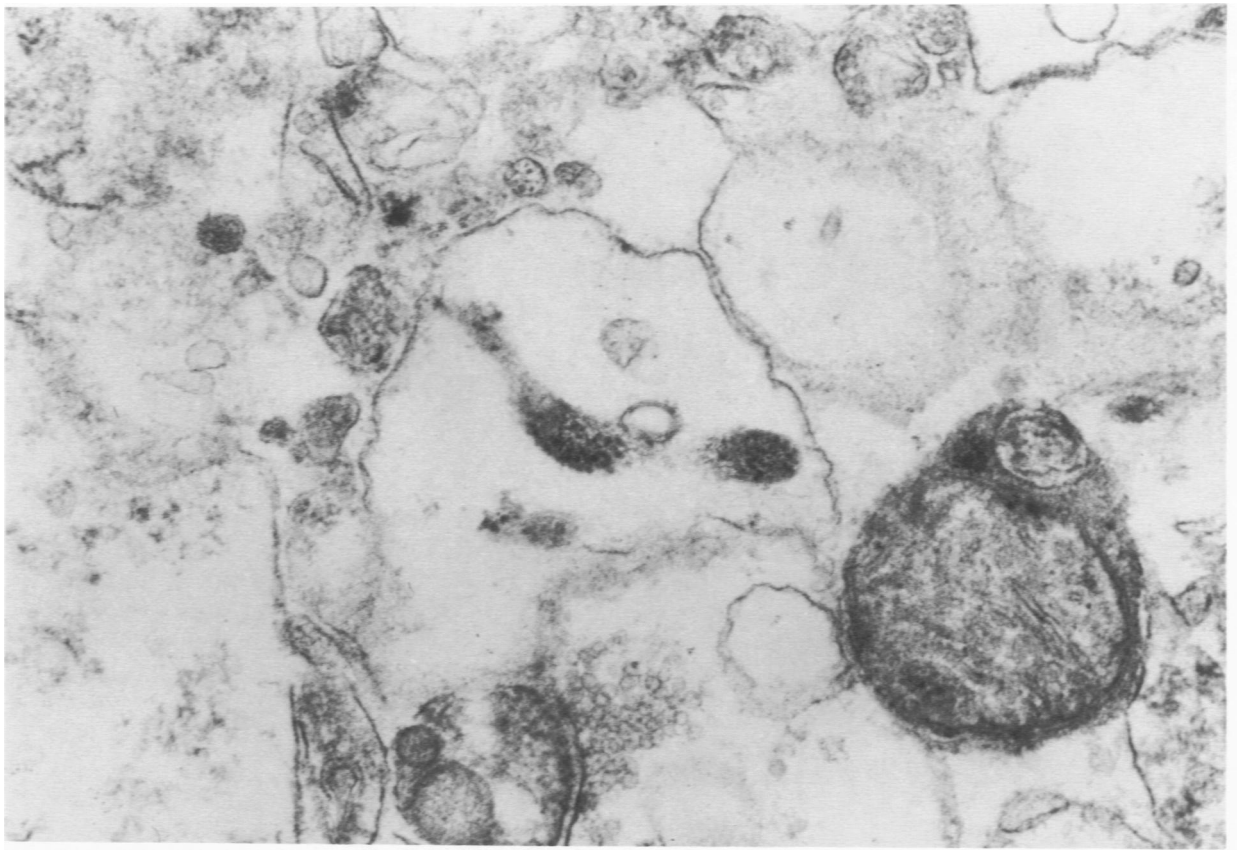
involved the superior aspects of both cerebral hemispheres with extension into the brain stem. The neuropil was studded with vacuoles and showed a prominent inflammatory infiltrate of polymorphonuclear leukocytes and mononuclear cells (Figure 1). The pyramidal neurons of the hippocampi showed striking vacuolar degeneration some distance from the primary site of inflammatory response (Figure 2). Sections of cerebellum showed no pathologic alteration.

Histologic examination of brain tissues obtained from rats 25 days after intracranial inoculation revealed minimal pathologic alteration (Figure 14). There was vacuolization of a few scattered neurons. This paucity of pathologic findings occurred in spite of persistent high titers of *Spiroplasma* organisms in brains from similarly infected 25-day-old animals (see Table 1). Brain tissues obtained from a companion group of rats inoculated intracranially with sterile culture medium and examined at 14 and 25 days after inoculation showed no pathologic change.

Ultrastructural Study

Electron-microscopic study of brain tissues from rats examined 14 days after intracranial inoculation readily revealed spiroplasma in vacuoles (Figures 7–9) and neuronal processes within the neuropil (Figure 10). Spiroplasmas were recognized in tissue by comparison with spiroplasmas in a centrifuged pellet of an *in vitro* culture (Figures 12 and 13). In both tissue and pellet, the spiroplasmas were predominantly seen as filaments or crescent shapes. Membrane blebs were observed in the infected rat brain tissues in close association with the recognized organisms. Similar membranous blebs were seen by electron microscopy in the spiroplasma culture pellet. The presence of

Figure 10 — High-power electron micrograph of a neuropil adjacent to the vacuole demonstrated in Figure 7 shows spiroplasmas within a dilated neuronal process. (×40,000) **Figure 11** — A single helical form of *Spiroplasma* was observed within the 14-day-old infected rat brain. (×40,000)



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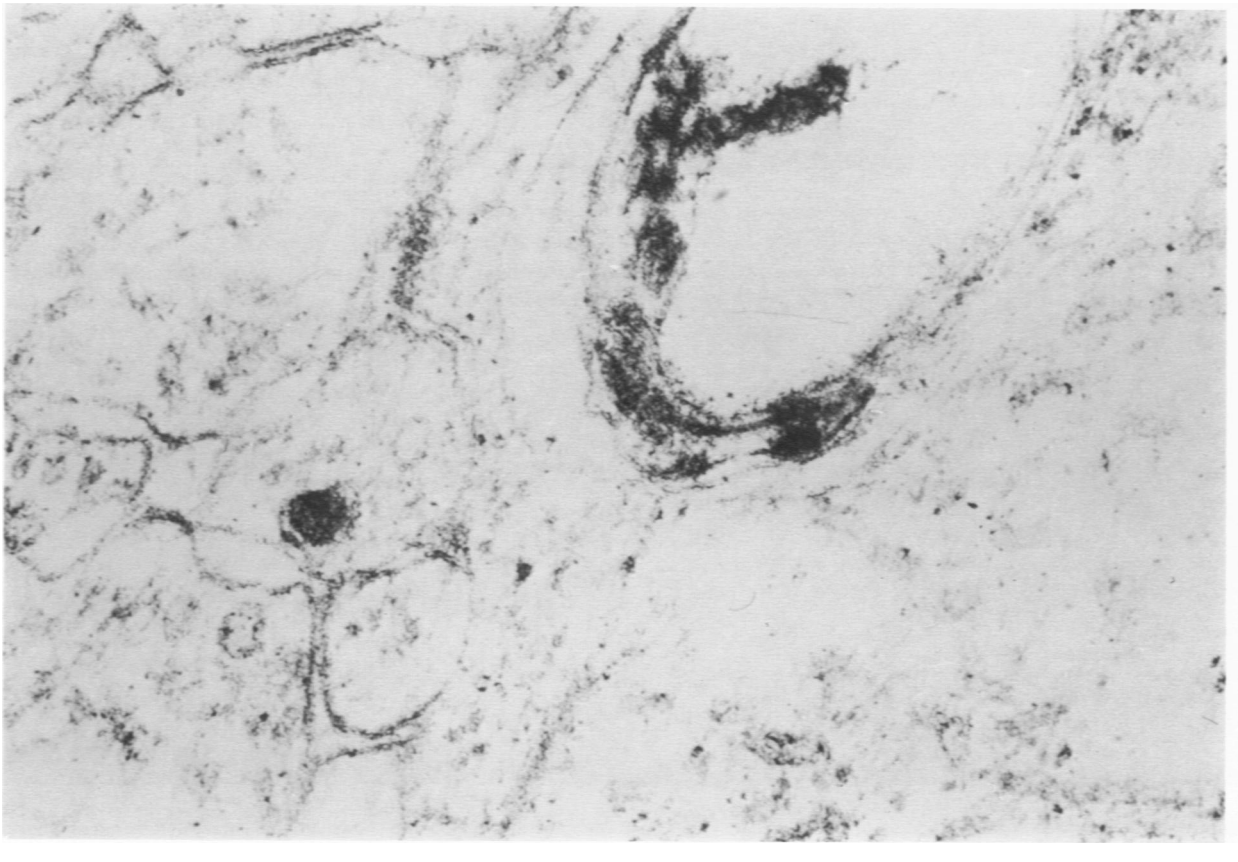

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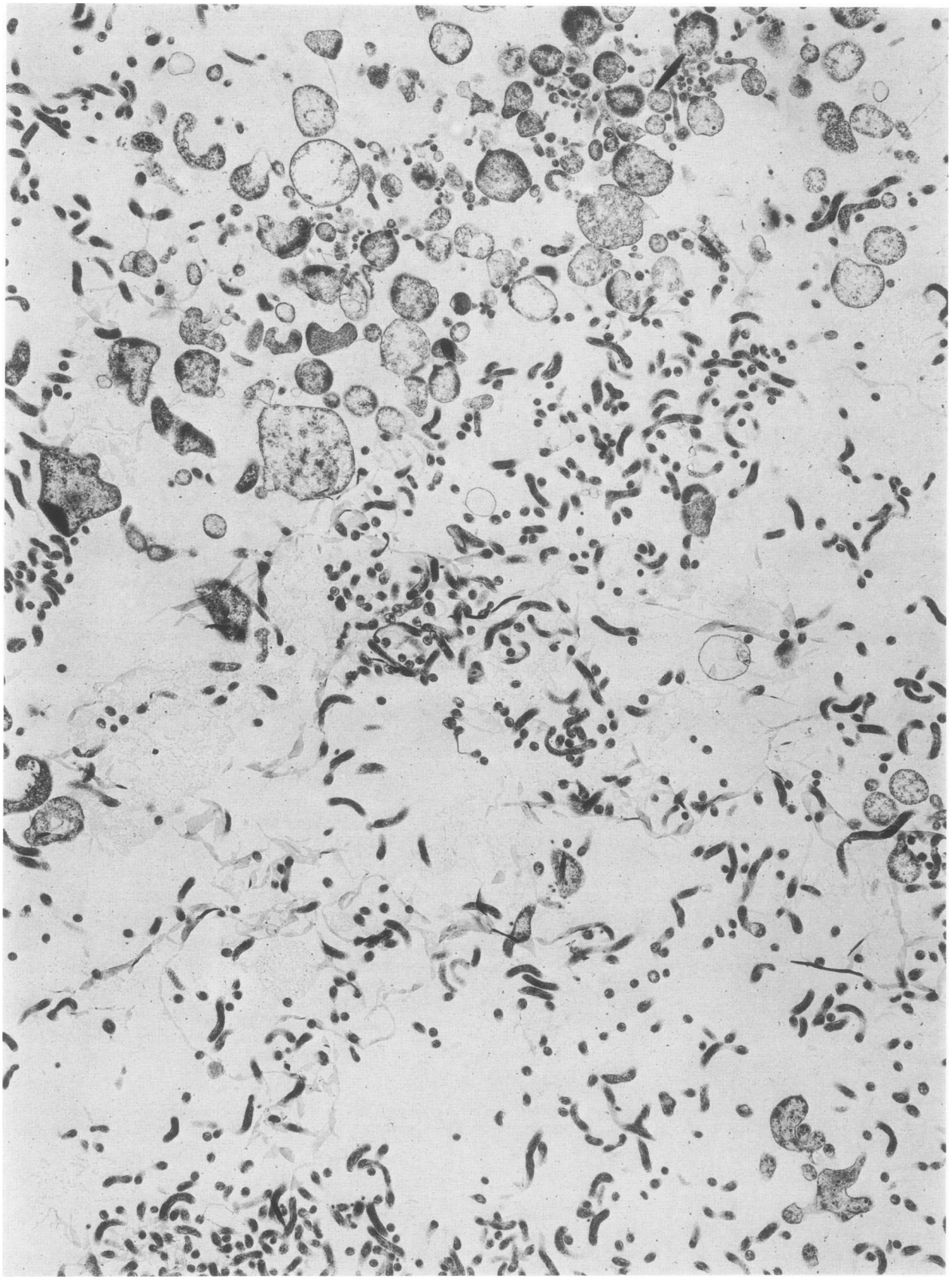


Figure 12 — Low-power electron micrograph of centrifuged pellet of GT-48 spiroplasma broth culture shows variable ultrastructural morphology of spiroplasmas *in vitro* with the presence of bleb forms. (×15,000)

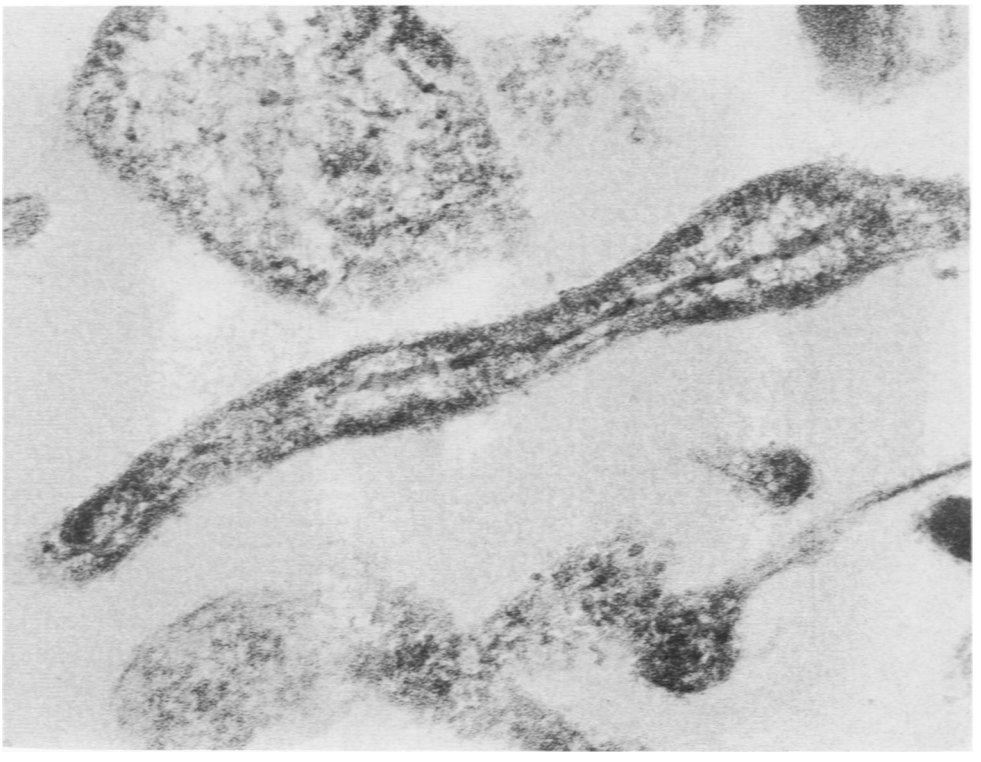


Figure 13 – Higher-magnification electron micrograph of spiroplasma broth culture shows filament-shaped and bleb forms of spiroplasmas. (×50,000)

spiroplasmas in the rat brain neuropil was often associated with breaks in adjacent host membranes (see Figure 8). A fortuitous section of cortex revealed a helical membranous structure representing a tightly coiled organism (Figure 11).

Electron microscopy of brain tissues obtained from rats 25 days after intracranial inoculation showed widespread dilatation of neuronal processes with focal accumulation of membranous debris (Figure 19). Spiroplasmas were not observed in these tissues after extensive search by electron microscopy. This apparent nonexistence of spiroplasmas occurred despite assay titers of 10^3–10^4 organisms/g of brain tissue from the same group of animals (see Table 1). One rat brain bioassay was negative for *Spiroplasma*. Electron-microscopic examination of brain from groups of rats examined beyond 25 days after inoculation have also revealed a lack of morphologic evidence of spiroplasmas in the rat neuropil despite their persistence in the tissues as determined by bioassay. This discrepancy has not been resolved as yet because long-term studies are incomplete.

Immunocytochemistry

Examination of PAP-stained paraffin sections obtained from rat brain 14 days after intracranial inoculation, following application of specific GT-48 antisera (as determined by metabolism inhibition test and positive staining of a centrifuged pellet of GT-48 broth culture), revealed immunostained bodies localized to the portions of brain tissue showing the microcystic encephalopathy (Figure 3). The brown pigment deposits of the PAP were seen within inflammatory cells, extracellularly, and within the vacuoles. Additionally, immunostaining was seen within the leptomeninges, the epithelial cells of the ependyma, and the choroid plexus. Examination of serial rat brain paraffin sections treated with preabsorbed GT-48 antisera or sections from rats previously inoculated intracranially with sterile culture medium showed no staining with PAP.

Examination of semithin sections of Epon-embedded rat brain tissue from these same 14-day-old infected animals treated with specific GT-48 antisera appeared to enhance localization of the spiroplasma antigen as determined by PAP staining (Figures 4–6). Furthermore, it was possible to confirm the reliability of the immunostaining through the examination of an adjacent serial section by electron microscopy. By this method the spiroplasma antigen was seen aligned along the periphery of the vacuoles. The immunostained *Spiroplasma* observed within the neuropil adjacent to the vacuoles was further documented by electronmicroscopy to be clearly within neuronal processes. Similar examination of infected brain tissues

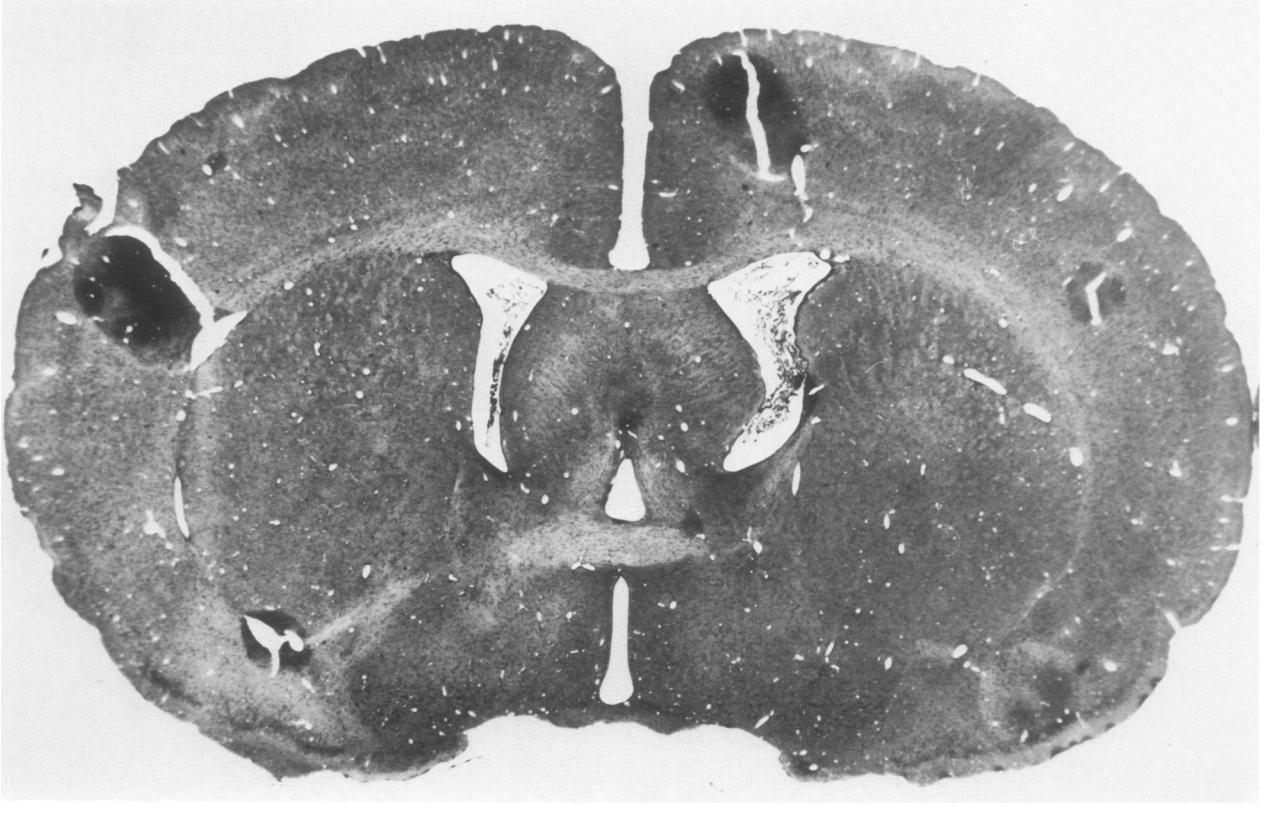


Figure 14—Coronal section of rat brain examined 25 days after intracranial inoculation of GT-48 spiroplasmas shows no significant pathologic alteration despite bioassay titers of 10^4 organisms/g of brain tissue (see Table 1). (H&E, ×50)

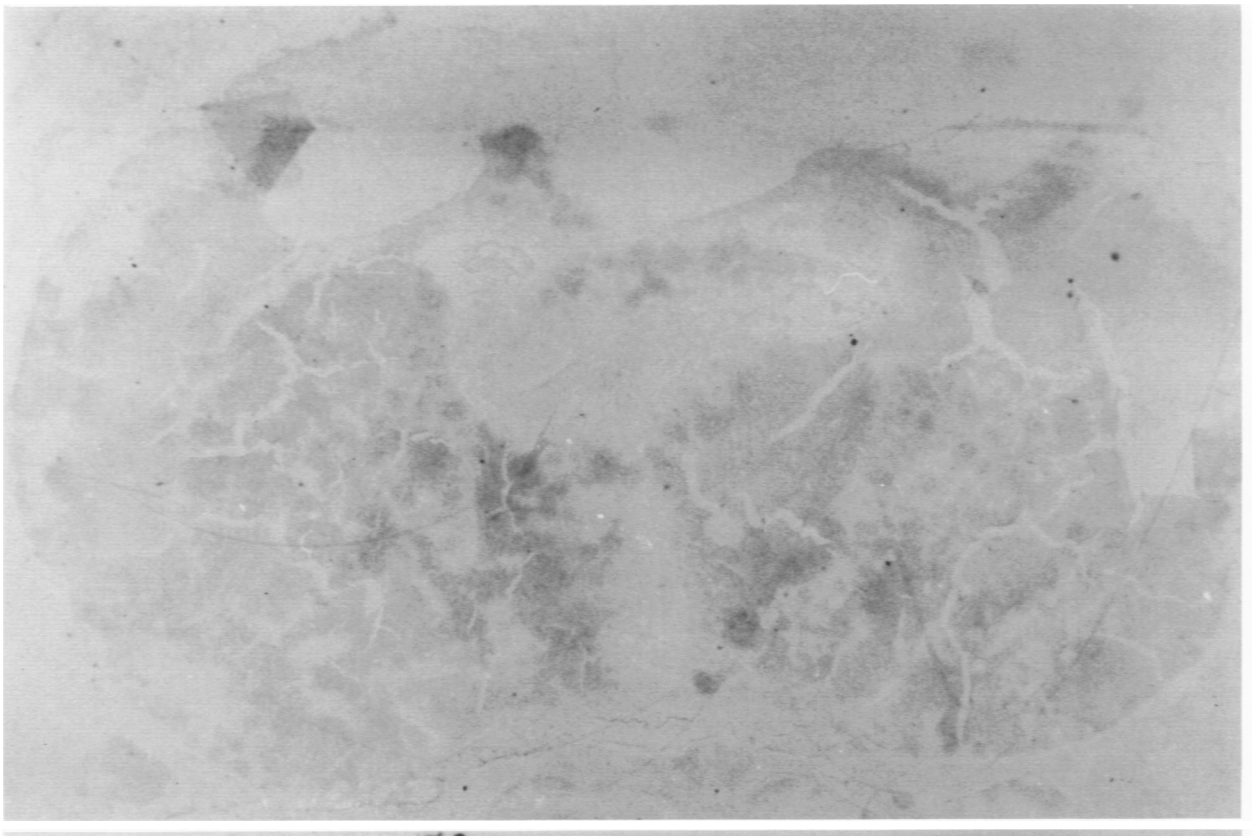
treated with preabsorbed GT-48 antisera or brain tissues from rats previously inoculated intracranially with sterile culture medium showed no PAP staining.

Examination of paraffin sections of brain tissues from rats 25 days after intracranial inoculation revealed considerable PAP staining. These findings were supported by the culture assays (see Table 1). The spiroplasma antigen was demonstrated in tissues that were histologically without pathologic reaction or inflammatory infiltrate. There was striking localization of the PAP staining to both superficial and deep gray matter (cerebral cortex and basal ganglia) (Figure 16). A serial section immunostained with preabsorbed GT-48 antisera is shown for comparison (see Figure 15). In the cerebellum immunostained bodies were observed in close association with Purkinje and granular cells. Similarly treated sections of hippocampus revealed immunostained bodies localized to the pyramidal neurons and to the granular cells of the dentate (Figures 17 and 18). In all instances the immunostaining was eliminated by treatment of the sections with antisera preabsorbed with GT-48 organisms. Sections of rat brain inoculated intracranially with sterile culture medium revealed no immunostaining when treated with specific GT-48 antisera and the PAP method.

Discussion

In this study we evaluated morphologic changes expressed in rat brain tissues by experimental spiroplasma infection in relation to microbiologic assay titers of the organisms. In the early stages of spiroplasma infection minimal pathologic changes were observed (7 days after intracranial inoculation). Microbiologic assay of those tissues showed that the spiroplasma infection spread rapidly throughout the brain tissues, reaching peak titers within 14 days following intracranial inoculation. At that time a prominent microcystic encephalitis was seen with influx of both polymorphonuclear and mononuclear inflammatory cells. At this stage, nonhelical but

Figure 15—PAP-stained serial section of rat brain as represented in Figure 14 following treatment with preabsorbed GT-48 antiserum as control. (×50) **Figure 16**—PAP-stained serial section of rat brain as represented in Figure 14 after treatment with specific GT-48 antisera shows immunostained bodies in the cortex and basal ganglia. (×50)



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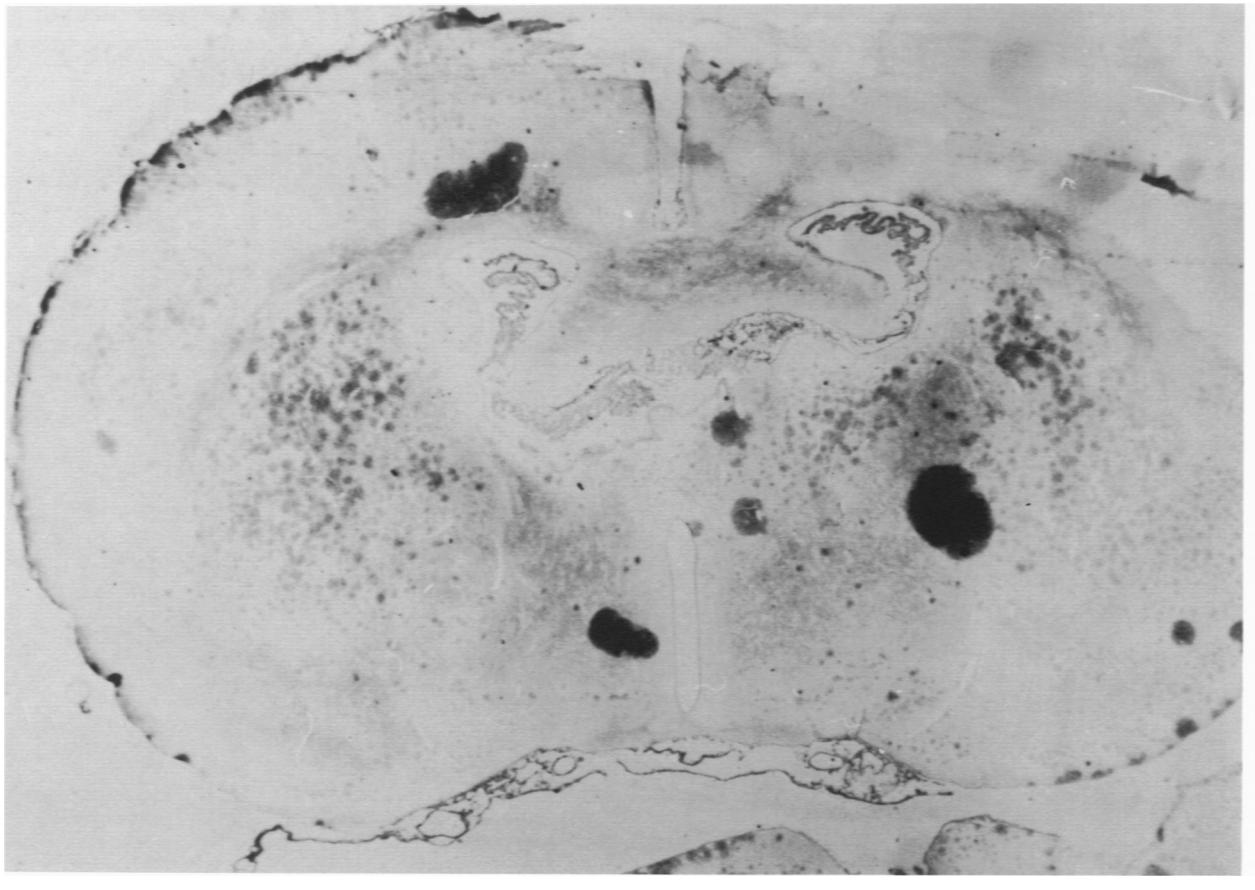

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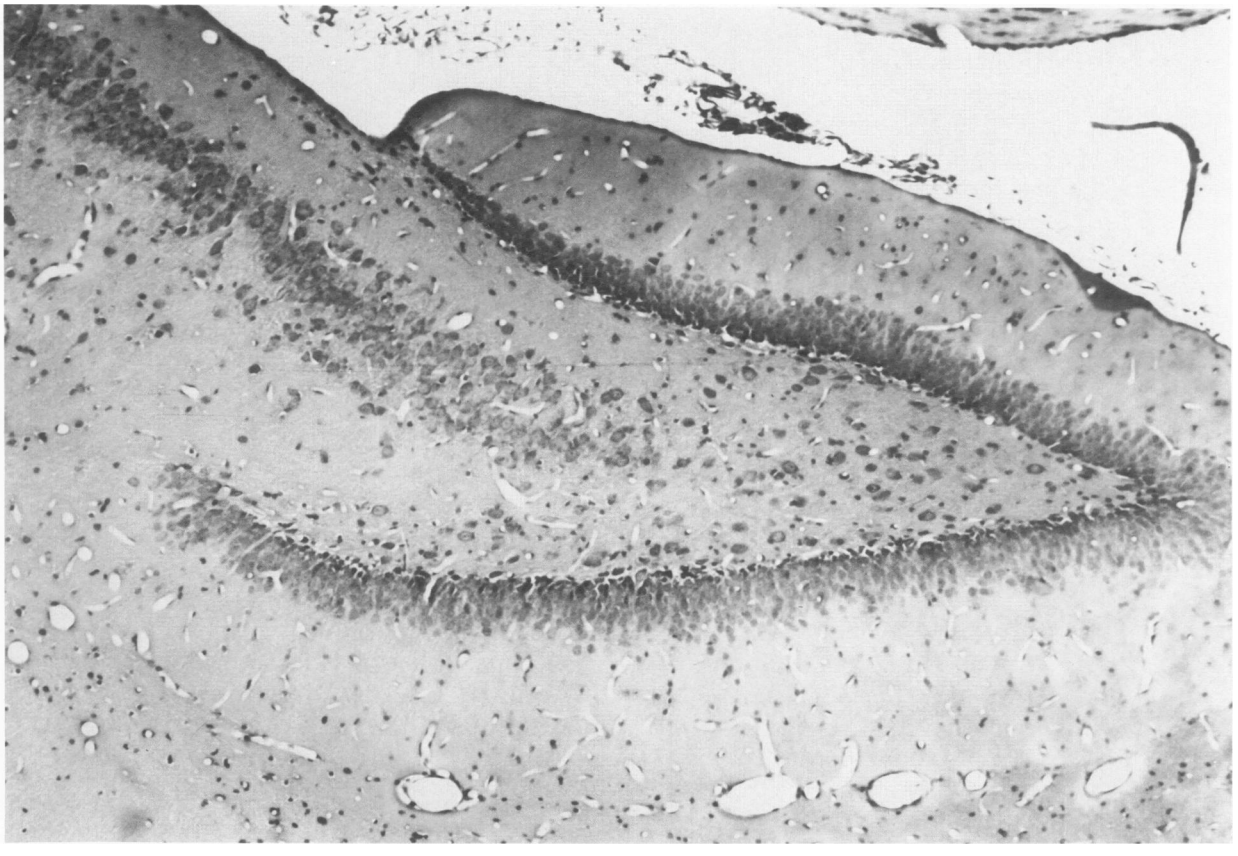

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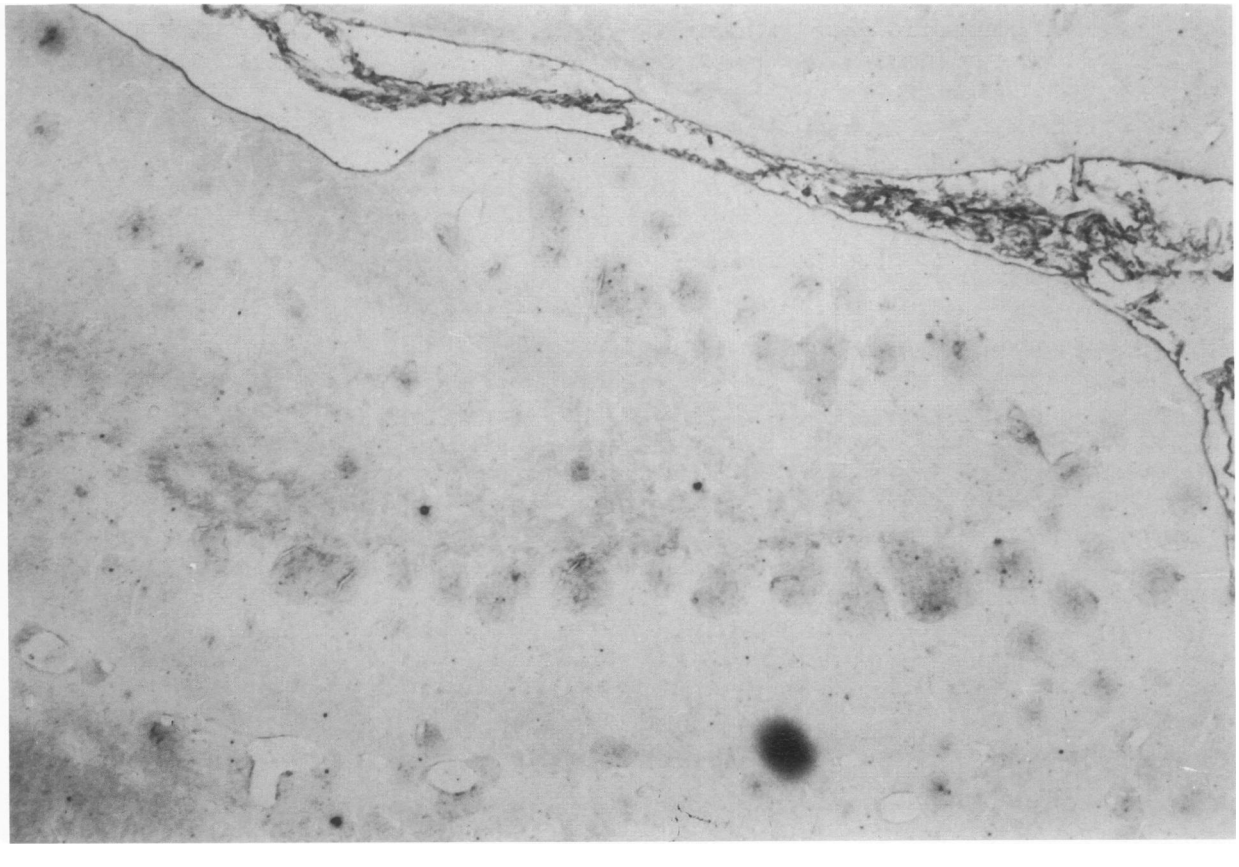

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crescent-shaped *Spiroplasma* organisms were easily identified by electronmicroscopy within the vacuoles and within neuronal processes in the rat neuropil. Immunocytochemical study confirmed the presence of spiroplasma within the diseased tissues, ie, within the microcysts and the meninges. Control preparations attested to the validity of these findings. At later stages of infection (25 days after intracranial inoculation and beyond) there was a distinct lack of inflammatory reaction with little visible pathologic alteration in spite of persistently elevated microbiologic assay titers of *Spiroplasma.* Immunocytochemical study of these tissues revealed significant amounts of spiroplasma antigen in both superficial and deep gray matter, areas which showed no histologic evidence of disease. Spiroplasmas were not visualized by electron microscopy in these brain tissues in late stages of infection even after extensive search at high magnifications.

Although spiroplasmas have been taxonomically classified with the mycoplasmas,[13] they are seen in this study within the cytoplasm of neurons. This suggests that spiroplasmas are capable of cell invasion unlike any known mycoplasma and presumably can migrate by intraneuronal transport. This phenomenon would account for the rapid spread of experimental spiroplasma infection throughout intracranially inoculated rat brain tissues.[14] This proposed transport mechanism is supported by the finding of spiroplasma antigen by immunocytochemistry in which spiroplasmas consistently localize to the superficial and deep gray matter. Presumably the organism eventually migrates to the neuronal parakaryon. High titers of spiroplasmas in the tissues without inflammatory response or pathologic alteration suggests a unique adaptation of the organism to the host. This peculiar host adaptation could be related to the intracellular localization of the organisms. The presence of microcystic change in the neuropil also appears to be an integral part of the tissue response to the spiroplasma supported by our finding of spiroplasmas in close association with the vacuoles. Membranous bleb forms of spiroplasmas were seen in both centrifuged pellets of spiroplasma broth culture and rat brain tissues examined at peak infection. This morphologic variability of the organism could explain why spiroplasma cannot be identified by electron microscopy within the neuropil in later stages of the infection. Presumably these membranous bleb forms of the organism blend into the background of the rat neuropil and are undetectable by current electron-microscopic techniques.

Tissue response to experimental spiroplasma infection in the suckling rat brain resembles the spongiform degenerative brain diseases of both man and other mammals. Initially this comparison was made because spiroplasmalike inclusions were seen by electron microscopy in brain tissues from several cases of Creutzfeldt-Jakob disease (CJD).[15-19] Further interest in this comparison was supported by the discovery of specific strains of *S mirum* that have an optimum growth temperature of 30–37 C and produce an acute or persistent brain infection in small mammals.[5,7-9,20] The experimental spiroplasma brain infection is characterized by at least a transient microcystic encephalopathy. Although this tissue reaction disappears, spiroplasmas can still be demonstrated in the rat brains in abundance by both microbiologic assay and immunocytochemistry. This unique adaptation of *Spiroplasma* to the rat brain is similar to the lack of inflammatory tissue response to the transmissable agent of CJD. Furthermore, the experimental spiroplasma infection is shown to localize to gray matter; a point of interest when one considers that CJD is a gray matter disease. Spiroplasmas cannot be recognized by electron microscopy in rat brain in late stages of infection, suggesting a corollary with the elusiveness of the transmissable agent of CJD. Indeed, the membrane bleb forms of spiroplasma as seen by electron microscopy in this study closely resemble the membranous profiles commonly associated with CJD infection. Even the 4-nm paired fibrils recently found consistently in CJD tissues[20] closely resemble the fibrils observed when the membranes of spiroplasmas are disrupted by nonionic detergent.[21,23]

This study represents the only in-depth morphologic examination of experimental spiroplasma infection in the rat brain. Clark[24] initially demonstrated that experimental spiroplasma infection produces a microcystic encephalopathy, but he was unable to find organisms in the infected brain tissues by electron microscopy. Since Clark's early work, cultivation of the organism on artificial media and more adequate microbiologic assays have enhanced our ability to correlate numbers of infectious units to corresponding morphologic changes. Subsequent studies have shown some resistance of *Spiroplasma* organisms to chemical factors[25] with some resemblance to the resis-

Figure 17 – H&E-stained section of infected rat hippocampus obtained from the same animal examined in Figure 14 shows no pathologic alteration other than minimal vacuolization of neurons. (×400) **Figure 18** – PAP-stained serial section represented in Figure 17 after treatment with specific GT-48 antisera shows localization of spiroplasma antigen to pyramidal neurons and granular cells of dentate. (×400)

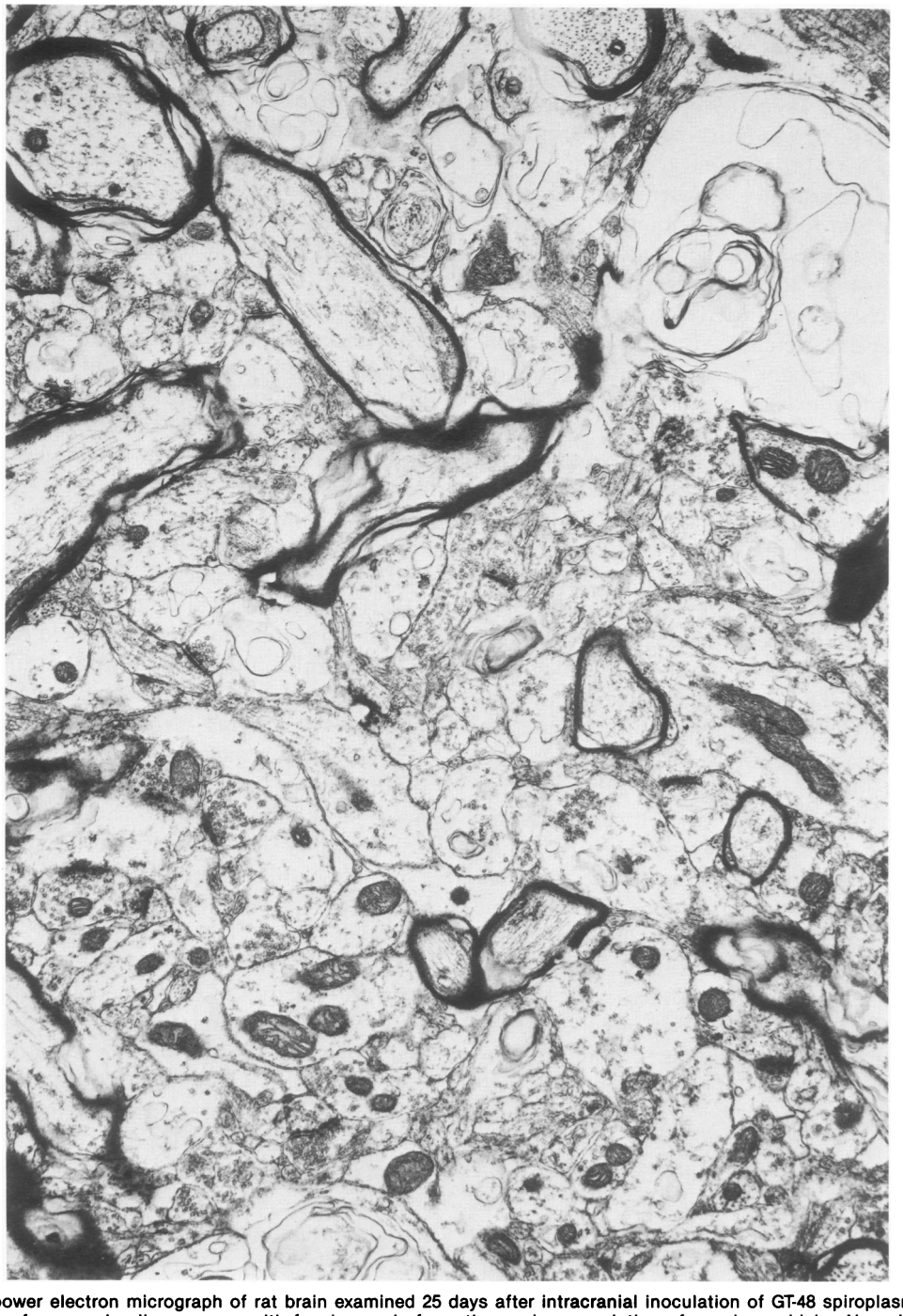

Figure 19—Low-power electron micrograph of rat brain examined 25 days after intracranial inoculation of GT-48 spiroplasms shows widespread dilatation of neuronal cell processes with focal vacuole formation and accumulation of membrane blebs. No spiroplasma forms were observed in these tissues after extensive search. (× 10,000)

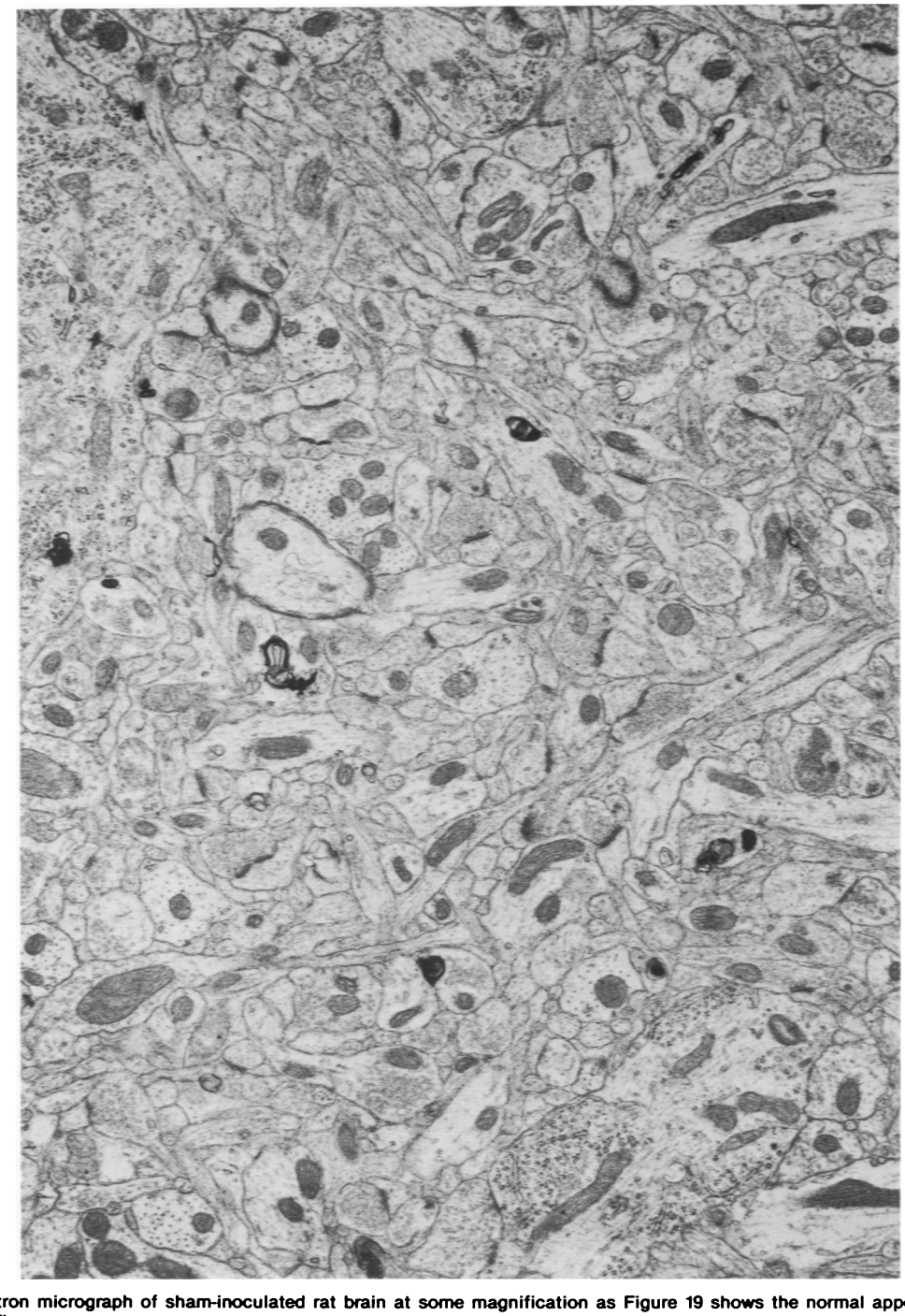

Figure 20 – Electron micrograph of sham-inoculated rat brain at some magnification as Figure 19 shows the normal appearance of neutrophil. (× 10,000)

tance expressed by the transmissable agent of CJD or scrapie.[26] Any differential in these studies could be accounted for by the fact that transmission experiments testing the infectivity of the CJD and scrapie agent were performed with the use of crude preparations of infectious materials. When repeated with more purified preparations, the agent's characteristics were shown to be more in line with conventional viruses, ie, the tobacco mosaic virus.[26] Nonetheless, current theories regarding the nature of the transmissable agent of scrapie and CJD have suggested that it is decidedly unusual and closely membrane-associated. We believe that the size and membranous nature of *Spiroplasma* organisms would best fit with the data available.

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