Identification of *Rickettsia rickettsii* in a Guinea Pig Model by Immunofluorescent and Electron Microscopic Techniques

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Moribund guinea pigs infected with *Richettsia rickettsii* were examined by necropsy, histology, immunofluorescence, electron microscopy, and serology. Untreated animals died at 9 and 10 days after inoculation. Animals given saline subcutaneously survived from 1 to 4 days longer. Prolonged survival was accompanied by more severe lesions: scrotal necrosis; infarction of ears; and swollen, hemorrhagic footpads, epididymis, and cremaster muscle. Histopathologic examination demonstrated that acute, necrotizing vasculitis, perivascular hemorrhage, and focal necrosis were more extensive. Direct immunofluorescence indicated many more rickettsiae in endothelium and vascular wall of saline recipients. Ultrastructurally, typical rickettsiae were present focally in the cytoplasm of endothelial and vascular smooth muscle cells. Cytopathology in infected and adjacent cells included swelling, mitochondrial enlargement with decrease in matrix density and loss of cristae, and increased pinocytosis. In addition, treated animals had more cytonecrosis, thrombosis, extravascular fibrin deposition, prominent inflammatory cells with polymorphonuclear phagocytosis of rickettsiae, and antibody production. (Am J Pathol 86:343–358, 1977)

THE LESIONS IN MAN caused by Rocky Mountain spotted fever rickettsial infection have been described in detail.[1-7] Lillie observed that the fundamental lesion of spotted fever is vascular and is characterized by endothelial swelling; occasional vascular occlusion, necrosis, or thrombosis; and perivascular cellular infiltration by polymorphonuclear leukocytes, lymphocytes, plasma cells, and macrophages.[5] Rickettsiae are present in endothelial and perithelial cells, including vascular smooth muscle cells. The consequences of this rickettsial tropism include maculopapular rash, petechiae and necrosis of skin, meningoencephalitis, interstitial myocarditis, interstitial pneumonitis, focal hepatic necrosis, mild glomerular hypercellularity, lymph node hyperplasia with sinus histiocytosis, and vasculitis in any organ but especially in epididymis, testis, and tunica vaginalis. The diagnosis of Rocky Mountain spotted fever is compounded by the great difficulty in identifying the organisms by using Giemsa, Pinkerton's, and other stains.

Guinea pigs are highly susceptible to infection with *Rickettsia rickettsii*,

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the etiologic agent of Rocky Mountain spotted fever. They become febrile on the fourth or fifth day after intraperitoneal inoculation and die on the ninth or tenth day with dehydration and hypovolemic shock. The pathologic findings include vasculitis with focal thrombosis and necrosis in scrotal skin, cremaster muscle, and epididymis; multifocal small hepatic infarcts; and congestion of splenic red pulp. Kidneys, epididymis, and testis of guinea pigs infected with *R. rickettsii* have been examined ultrastructurally. Renal lesions consist of mild endothelial swelling, active enlarged mesangial cells, and occasional irregular thickening of the lamina rara interna of the basement lamina.[8] No rickettsiae have been demonstrated in kidneys. Vessels of epididymis and testis first manifest endothelial swelling and mitochondrial enlargement with loss of cristae.[9] Later in the infection course, endothelial cells show more marked mitochondrial alterations and contain many pinocytotic vesicles at luminal and basal surfaces. Finally, gaps between and disappearance of endothelial cells occur with accumulation of platelets and fibrin. Perivascular inflammation and rickettsiae have been described but not illustrated.

Our ultrastructural study of guinea pig epididymis, cremaster muscle, and lymph node was undertaken to further elucidate the late stages of the Rocky Mountain spotted fever rickettsial infection. In order to allow the development of late lesions analogous to those seen in terminal human disease, we gave parenteral fluids to some infected guinea pigs. This treatment allowed protracted survival and was of value in considering the relation of the microorganisms to the progression of lesions. The superior demonstration of rickettsiae by immunofluorescence suggests that the technique might be applied to early diagnosis of human Rocky Mountain spotted fever.

Materials and Methods

Animals and Rickettsiae

Fifteen random-bred guinea pigs (500 to 650 g) from the Center for Disease Control (CDC) colony were inoculated intraperitoneally with 10^4 plaque-forming units of *Rickettsia rickettsii*, Sheila Smith strain, contained in 1.0 ml of pooled guinea pig 50% spleen-blood suspension diluted 1:50 in sucrose-phosphate-glutamate solution. This standard rickettsial strain was obtained from Dr. Charles Shepard, Leprosy and Rickettsia Branch, CDC, and further passed in guinea pigs to obtain uniform stock in 50% spleen-blood suspension. Ten animals (Group A) received no other treatment. Five animals (Group B) were maintained on saline given subcutaneously in 25-ml doses every 12 hours beginning at the onset of fever. Daily rectal temperatures, weights, and clinical observations were recorded. Animals were killed by cardiac exsanguination when they were moribund, usually at the time when body temperature fell from levels above 40 C to subnormal levels.

Light Microscopy

Specimens of scrotum, testis with epididymis and cremaster muscle, lymph node, spleen, liver, heart, lung, kidney, adrenal, and brain were collected during postmortem examination; they were fixed in 4% formaldehyde, dehydrated, embedded in paraffin, sectioned at 6 μ, and stained with hematoxylin and eosin and Giemsa stains. Selected tissues were stained with Pinkerton's stain.

Immunofluorescence

Samples of spleen, liver, lymph node, and testis were embedded in a polyethylene glycol compound (OCT, Ames Company, Division of Miles Laboratories, Elkhart, Ind.) and were frozen on dry ice. Frozen sections (4 to 6 μ) were cut, fixed in two changes of acetone for 10 minutes each, and stored at −20 C. Fluorescein-conjugated rabbit anti-*R. rickettsii* globulin fraction (obtained from Dr. Theodore Tzanibos, Bureau of Laboratories, CDC) was reacted with sections by the direct immunofluorescent technique for 30 minutes in a moist chamber at room temperature. The sections were then washed for 30 minutes in phosphate-buffered saline, dipped in distilled water, allowed to air dry, and mounted under No. 1 thinness coverslips in mounting media containing 90% glycerol and 10% phosphate-buffered saline. This conjugate had been shown to stain *R. rickettsii* antigen in infected yolk sacs and tick hemolymph. Uninfected tissue and tissue infected with *Neisseria meningitidis* did not take up the immunofluorescent antibody. Preincubation of sections from infected tissues with unlabeled antiserum to *R. rickettsii* markedly diminished staining as compared with preincubation with serum containing no antibodies to *R. rickettsii*. Nonspecific fluorescence was never observed, and absorption of conjugate was never required.

Electron Microscopy

One-cubic millimeter blocks of epididymis, cremaster muscle, and lymph node were dissected and fixed in 2.5% phosphate-buffered glutaraldehyde for 4 hours. Tissues were postfixed in 1% osmium tetroxide, dehydrated in graded alcohol concentrations, embedded in a mixture of Epon and Araldite,[10] sectioned, and stained with uranyl acetate and lead citrate.[11] Sections were examined in Philips 200 or 300 or JEM 100B electron microscopes.

Serology

Sera from all of the infected guinea pigs which had been maintained on parenteral saline were tested by complement-fixation [12] and microagglutination.[13] Sera from 3 untreated moribund animals were tested in the same way, and 4 were tested by complement fixation only. Serologic testing for antibodies to *R. rickettsii* was performed in the Leprosy and Rickettsia Branch, Virology Division, CDC, Atlanta.

Results

Clinical and Necropsy Observations

All susceptible guinea pigs developed a sustained fever ($\geq$ 39.8 C) between Days 4 and 6 after inoculation, the majority starting on Day 4. Scrotal reactions, characterized initially by erythema and edema and later by lividity and brawny edema, were observed in both groups of animals. Onset of this scrotal reaction occurred between 7 and 9 days after in-

oculation. Other clinical findings included mild-to-severe conjunctival erythema and crusted exudate, and occasional lividity of ears and footpads. Animals of Group A were moribund on Day 9 or 10 (Table 1). Saline recipients, which were moribund after Day 11, uniformly manifested severe conjunctivitis with closed eyes due to palpebral edema; autoamputation of purplish, crusted ears; severe livid edema of footpads; and severe scrotal reaction which in most animals had progressed to epidermal necrosis.

At necropsy, lesions were consistently found in the same organs and tissues in all animals. There were severe hemorrhagic epididymitis and cremasteric myositis, multiple ischemic hepatic infarcts, and markedly enlarged, reddish lymph nodes. Those animals sacrificed on Days 11, 12 and 13 exhibited more changes in a quantitative sense, but the character and location of lesions were the same.

Brightfield and Immunofluorescent Microscopic Observations

Microscopic lesions in testis, epididymis, and cremaster muscle of guinea pigs dying on Days 9 or 10 (Group A) were compared with those in animals which had received supportive saline therapy and were sacrificed when moribund 11, 12, or 13 days after inoculation (Group B). Animals in the latter group had more advanced lesions; they had necrotizing vasculitis (Figure 1) with intraluminal and extraluminal fibrin deposition, regional necroses of epididymis, and focal cremasteric rhabdomyolysis, in

Table 1—Response of Guinea Pigs to Inoculation With *R. rickettsii*

	Animal No.	Day of death	Mode of death
Group A—no saline treatment			
	1	9	S
	2	10	S
	3	10	S
	4	9	E
	5	9	S
	6	9	S
	7	9	S
	8	—	—
	9	10	S
	10	—	—
Group B—saline treatment			
	1	11	S
	2	12	S
	3	12	S
	4	13	S
	5	11	S

S = Animal sacrificed when in hypothermic, moribund state; E = animal expired; — = animal remained afebrile and well.

addition to the perivasculitis and hemorrhages which were present in the animals of Group A dying on Days 9 and 10. Similar infarcts were seen in ears and skin as well. Additionally, multifocal deposition of fibrin was noted in the sinusoids of the splenic red pulp, periarteriolar lymphocytic sheaths, and veins, and there was marked dilatation of the draining sinuses with erythrophagocytosis in lymph nodes of both groups of animals.

Rickettsiae were observed in the spleen, epididymis, and lymph nodes of all infected guinea pigs by immunofluorescence performed on frozen sections. It was not possible to determine in which splenic cells the rickettsiae were growing; however, histologic detail was outlined in epididymis by a blue-white tissue autofluorescence, and it was clear that rickettsiae were limited to vascular endothelium and other layers of vessel walls (Figure 2). Rickettsiae were present in lymph nodes in small numbers; they appeared to be in reticuloendothelial cells lining the sinuses and endothelium of capillaries.

There were many more microorganisms in the epididymis and spleen of animals which had received saline (and had lived several days longer) than in tissues of the other animals (Figure 3). In some epididymal foci in saline-treated animals, entire endothelial profiles were outlined by immunofluorescent rickettsiae. These immunofluorescent organisms were morphologically typical of rickettsiae; as expected in the random planes of section, some organisms assumed diplobaccillary configuration (longitudinal plane) and others were more coccoid (transverse plane). Organisms were always more difficult to find and identify when Giemsa and Pinkerton's stains were used, and consequently their numbers always seemed smaller and their identification more equivocal.

Ultrastructural Observations

Rickettsial infection in all target organs was focal, but infected cells often contained large numbers of clustered organisms. Rickettsiae were observed in vascular smooth muscle cells (Figure 4), in the thin, delicate endothelial cells of epididymis, cremaster, and lymph node capillaries (Figure 5), and in the thicker endothelial cells of other lymph node vessels. Organisms were also present within polymorphonuclear leukocytes (PMNs). Rickettsiae had an electron-dense cell wall, sometimes showing fine surface projections like Gram-negative bacteria. Their cell membrane was usually separated from the cell wall by an electron-lucent space (fixation artifact). The interior of the rickettsial cells contained numerous ribosomes, strands of electron-dense material (consistent with prokaryotic chromatin), and lucent foci.

These rickettsiae lay free within the cytosol of smooth muscle and endothelial cells; they were never enclosed by a membrane, but usually were surrounded by an electron-lucent halo of relatively uniform thickness. No organisms were unequivocally found in nuclei, although several were seen in cytoplasmic invaginations into nuclei (Figure 6). In some cases, rickettsiae were present in cytoplasm abutting the nuclear envelope.

The morphology of extracellular organisms was usually characteristic, so that their identification amidst cellular debris was possible. There were more extracellular organisms in regions of complete cytonecrosis, and these often assumed a rounded, swollen shape, possibly representing osmotic damage. Some damaged rickettsiae had irregularly accordionized cell walls, with marked retraction of cell membranes and increased density of cell contents (Figure 7). Stages of binary fission, the replicative mode of rickettsiae, were identified only intracytoplasmically (Figure 8).

Host Cell Cytopathology

Cytopathologic lesions affected mitochondria, rough endoplasmic reticulum, vesicles, vacuoles, cytosol, nuclear envelope, and nuclei. Of these, the mitochondria of endothelium, vascular smooth muscle, and adjacent perivascular cells showed the most striking alterations. Some mitochondria became markedly swollen with decreased mitochondrial matrix density and decreased number and concentration of cristae (Figure 9). A spectrum from normal structure to this degree of dissolution was represented in all infected tissues. These severe changes occurred in some cells in which there was no other evidence of infection; a spatial relation to the presence of rickettsiae was difficult to ascertain in a single plane of section.

Dilatation of rough endoplasmic reticulum occurred in some infected cells, and in some cases this was closely associated with the presence of rickettsiae. Cytoplasmic vacuoles were also observed and may have represented extreme examples of either mitochondrial swelling or dilatation of endoplasmic reticulum. Increased pinocytosis occurred at the external and luminal surfaces of endothelium and at all cell surfaces of vascular smooth muscle and perithelial cells. The alternate necrotic pathways were in evidence: some cells exhibited cytosol rarefaction with organelle destruction, and other cells underwent cytoplasmic condensation.[14] Additional focal cytopathic changes included nuclear pyknosis, dilatation of nuclear envelope, and apparent increase in "cytoplasmic residual bodies." Endothelial cell necrosis was usually accompanied by luminal thrombosis. In many foci there was endothelial cell dropout (Figure 10) which apparently resulted from the detachment and sweeping away of injured endothelial

cells into the blood stream. Late in the course of infection, large foci of disorganized necrotic cell debris totally effaced organ architecture in those animals given saline therapy; these foci consisted only of fibrin, amorphous debris, collagen, and microorganisms.

Inflammation

Cellular inflammatory response to the infection was vigorous. The massive influx of cells included PMNs, macrophages, and lymphocytes. PMN leukocytes infiltrated perivascular and intramural locations. Some contained phagocytosed rickettsiae in various stages of degeneration within phagolysosomes (Figure 11). Monocytes and activated macrophages were present in capillary lumina and in perivascular and intramural locations. Lymphocytes were likewise observed in similar perivascular and intramural foci. Erythrocytes were not only packed in capillary lumina but also distributed in extravascular spaces. In thrombotic foci and sites of extravasation, many erythrocytes exhibited varying degrees of hemolysis. In these sites of exudation, amorphous material rich in fibrin filled the spaces between parenchymal cells, inflammatory cells, and the cellular debris.

Serologic Response

The prolonged survival of animals which had received saline allowed the development of higher antibody titers to *R. rickettsii* than in the guinea pigs which died earlier in the course of disease (Table 2). The microagglutination test gave higher antibody titers than the complement-fixation test. In any case, the development of antibody was not life-saving.

Table 2—Serologic Response of Guinea Pigs Infected With *R. rickettsii*

	CF	MA
Group A—infected, no supportive treatment (7 of 10 animals bled and tested)	<8	8
	<8	32
	<8	16
	<8	NT
	<8	NT
	<8	NT
	<8	NT
Group B—infected, saline 25 ml every 12 hours, beginning at onset of fever (5 of 5 animals bled and tested)	<8	64
	<8	>256
	64	256
	64	128
	<8	128

CF = Reciprocal of complement fixation titer at time of sacrifice when moribund; MA = reciprocal of microagglutination titer at time of sacrifice when moribund; NT = not tested.

Discussion

The administration of fluid and electrolytes to febrile, anorectic guinea pigs infected with *R. rickettsii* prolonged life and suggested the importance of hypovolemia and altered sodium metabolism in fatal experimental Rocky Mountain spotted fever. Aikawa and Harrell [15] previously demonstrated weight loss and hyponatremia in infected guinea pigs. Instead of reversing the pathophysiology of rickettsial cell injury, however, the saline administration and prolongation of life allowed further replication of rickettsiae, further progression of cytopathology, and superimposition of thrombosis, ischemia, and anoxic necrosis.

This amplification and progression of pathologic processes enabled us to extend our investigation of host–parasite relationships and to explore the nature of terminal lesions. It was anticipated that findings would be comparable to those seen in fatal human cases receiving only nonspecific supportive care. The goal of relating the presence of rickettsiae to ultrastructural cytopathology was partially fulfilled. The cell types which were shown to be infected were the same types which exhibited specific organelle cytopathology, especially progressive changes in mitochondria. However, direct demonstration of rickettsial infection of each damaged cell was not obtained, and the question of whether a "soluble" factor might spread destruction beyond the location of organisms remains unanswered. The presence of organellar lesions in cells adjacent to infected cells implies this kind of extension, but in sites of massive inflammatory influx, parenchymal cell damage may be somewhat indiscriminant.

The striking early cytopathic effects in mitochondria may have been related to deficient adenosine triphosphate (ATP) production by rickettsiae and their consequent "energy parasitism."[16] Precise biochemical mechanisms by which rickettsia might usurp mitochondria-produced ATP and thereby cause structural damage to mitochondria are unclear. Dilated cisternae of rough endoplasmic reticulum, increased pinocytosis, and intracytoplasmic rarefaction may all reflect an influx into infected cells of water—intracellular edema. Theoretically, a deficiency of ATP could cause dysfunction of the sodium–potassium pump, which could result in the accumulation of intracellular sodium ions and consequently water. On the other hand, these ultrastructural cytopathic changes may be manifestations of common necrotic pathways, and therefore nonspecific.

Mammalian cell infection by *R. rickettsii* is intracytoplasmic, with no membranous separation of microorganisms from the cytosol. This relationship allows free entry to nutrients and exit of waste products from the rickettsial cell through its permeable cell wall. Moreover, this intimate relationship permits the rickettsia to evade host cell defenses. The absence

of an enclosing membrane barrier precludes heterophagy by the host cell and favors survival of the rickettsia with consequent necrosis of the host cell.

It is difficult to interpret these ultrastructural observations to prove or disprove any of the commonly suggested mechanisms involved in the pathogenesis of rickettsial disease. It still is not known whether the observed destructive changes result from a) energy parasitism, b) rickettsial competition for host cell substrates, c) endotoxin or other toxic rickettsial products, or d) immunopathologic mechanisms. For example, one suggestion regarding the role of immunopathology in Rocky Mountain spotted fever depends solely upon immunofluorescent demonstration of immunoglobulins, fibrin, and complement in late inflammatory lesions.[9] This kind of presumptive evidence should be interpreted more conservatively in light of the present demonstration of frank vascular damage and nonspecific extravasation of plasma proteins into lesion sites.

The host's inflammatory response and serologic response were not sufficient either to diminish the numbers of organisms or to reverse the pathophysiologic effects of endothelial/vascular injury. There was morphologic evidence of phagolysosomal degradation of rickettsiae by PMNs and macrophages, but this was not sufficient to result in a decrease in number of rickettsiae observed in the lesions.

The movement of infected endothelial cells may play a role in the spread of infection within a vertebrate host. Injury and detachment of infected endothelial cells and their entry into the circulation suggests that there may be an important intraendothelial cell rickettsemia.

Finally, the superior histologic demonstration of rickettsiae by immunofluorescence in frozen sections of guinea pig organs, as compared with that obtained with Giemsa or Pinkerton's stains, leads us to consider the possibilities of a rapid diagnostic test of human infection. The sensitivity and specificity of the immunofluorescence method might allow rapid, early diagnosis via skin biopsy; a trial of this approach is warranted.

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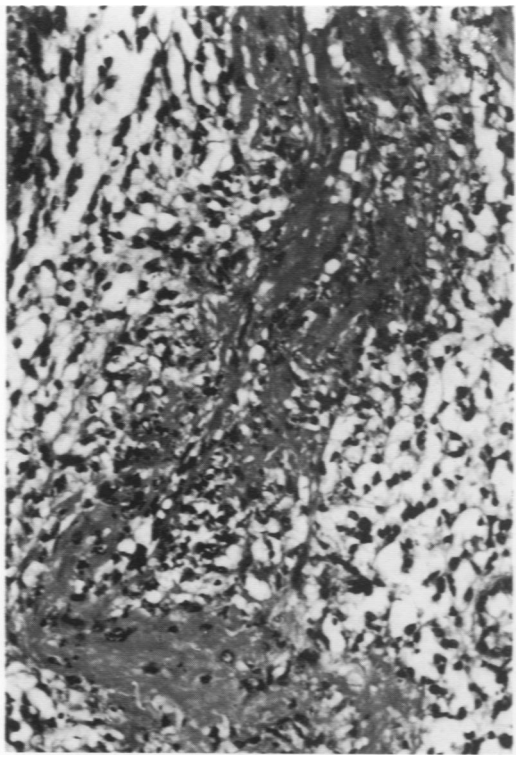
Legends for Figures

Figure 1—Epididymal vessel from guinea pig infected with *R. rickettsii* and maintained with saline treatment until sacrificed when moribund on Day 13. Artery in center of field has undergone necrosis with luminal, intramural, and perivascular deposition of fibrin. (H&E, × 140)

Figure 2—Epididymal vessel from guinea pig infected with *R. rickettsii* and sacrificed when moribund on Day 10. Specifically immunofluorescent rickettsiae are present in the endothelium (three rickettsiae at *arrow*) and vessel wall. (× 430)

Figure 3—Epididymal vessel from guinea pig infected with *R. rickettsii* and maintained with saline treatment until sacrificed when moribund on Day 13. Large numbers of immunofluorescent rickettsiae are present in the endothelium and other layers of vascular walls. (× 430)

Figure 4—Vessel from lymph node of guinea pig infected with *R. rickettsii* and maintained with saline treatment until sacrificed when moribund on Day 13. Vascular smooth muscle cells contain clusters of typical rickettsiae surrounded by clear halos. In this area, cells are in rather good condition despite the presence of large numbers of organisms. (× 13,400)



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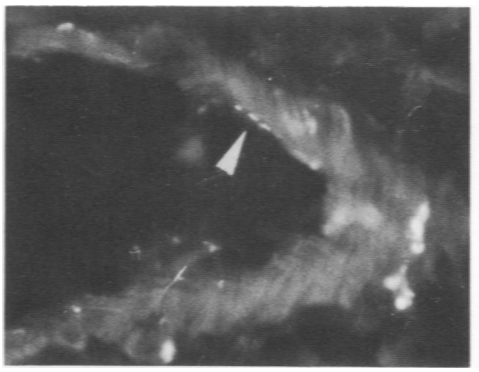

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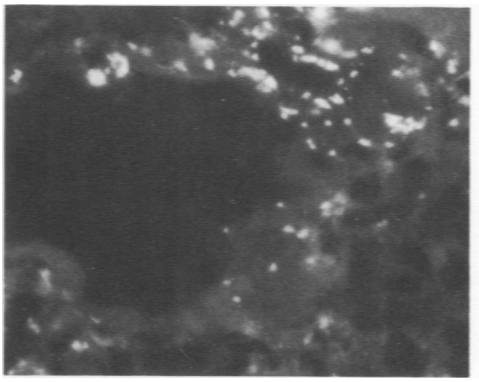

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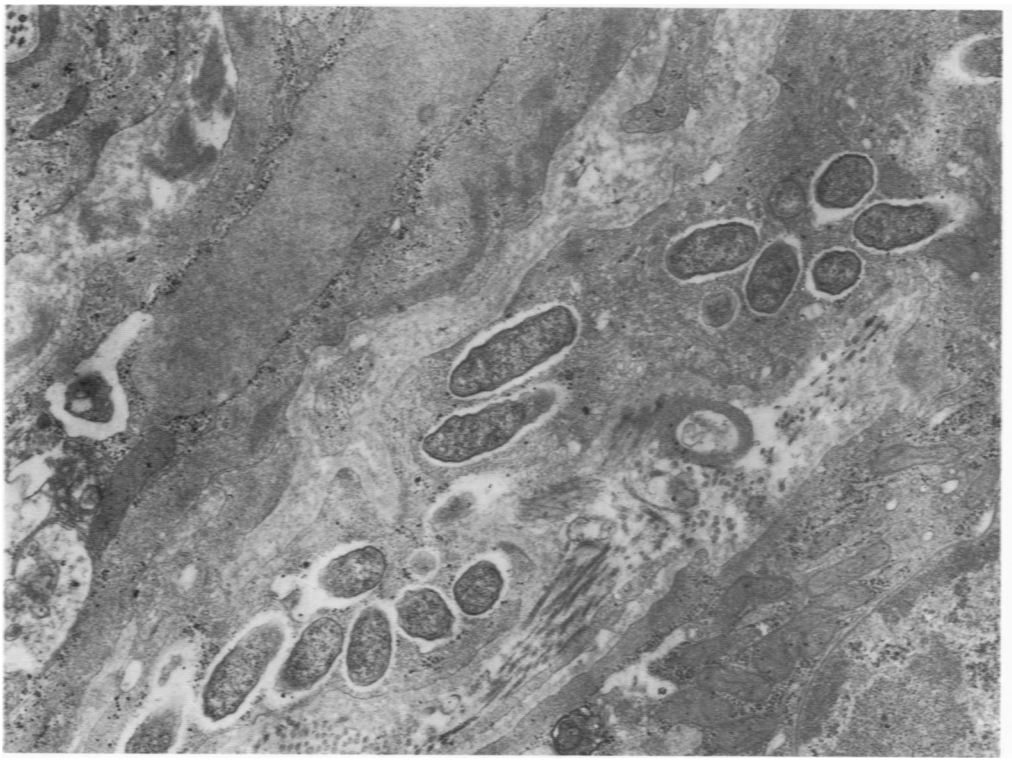

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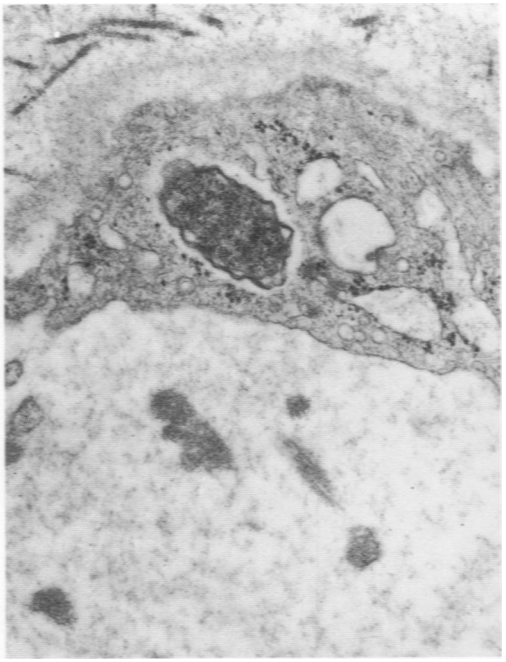
Figure 5—Capillary in testis of guines pig infected with *R. rickettsii.* A single rickettsial cell is present in this thin, delicate endothelial cell. Note fibrin in capillary lumen. (× 26,400)

Figure 6—Epididymis of guinea pig infected with *R. rickettsii* and maintained with saline treatment until sacrificed when moribund on Day 11. "Intranuclear" rickettsiae are actually in cytoplasmic invaginations separated from nucleoplasm by nuclear envelope. (× 29,600)

Figure 7—Epididymis of guinea pig infected with *R. rickettsii* and maintained with saline treatment until sacrificed when moribund on Day 11. This rickettsial cell exhibits evidence of injury: accordionization of cell wall, excess retraction of cell membrane from cell wall, and shrunken, dense interior. (× 32,500)

Figure 8—Testis of guinea pig infected with *R. rickettsii.* Vascular smooth muscle cell contains two pairs of rickettsiae in stages of binary fission. (× 26,400)



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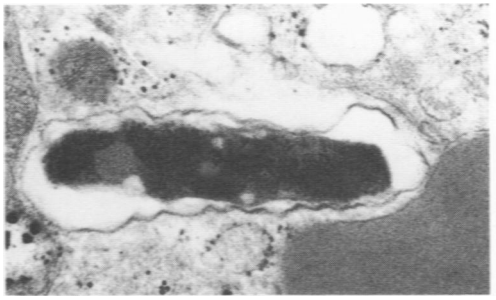

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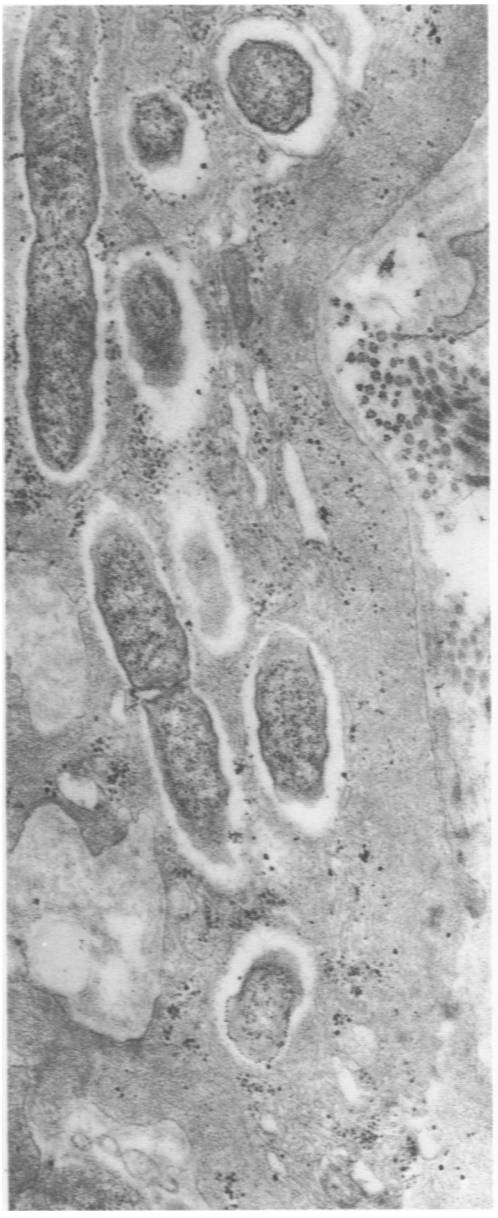

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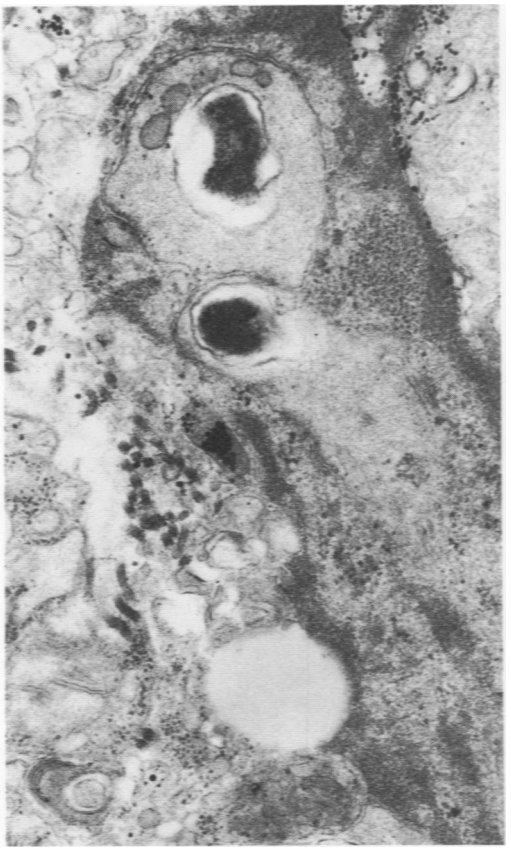

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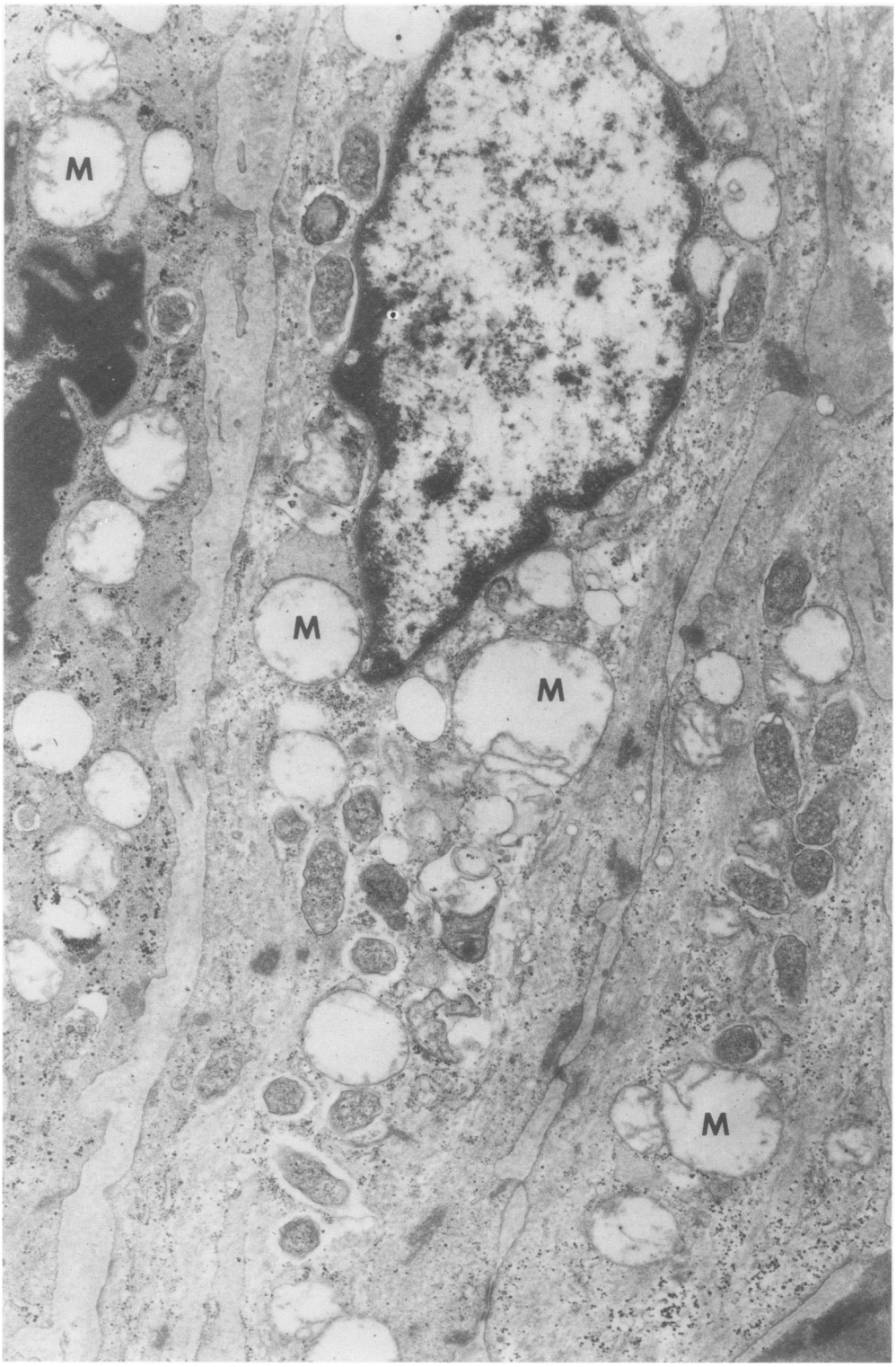

Figure 9—Epididymis of guinea pig infected with *R. rickettsii.* Vascular smooth muscle cells contain clusters of rickettsiae and markedly swollen, degenerated mitochondria (*M*). (× 15,900)

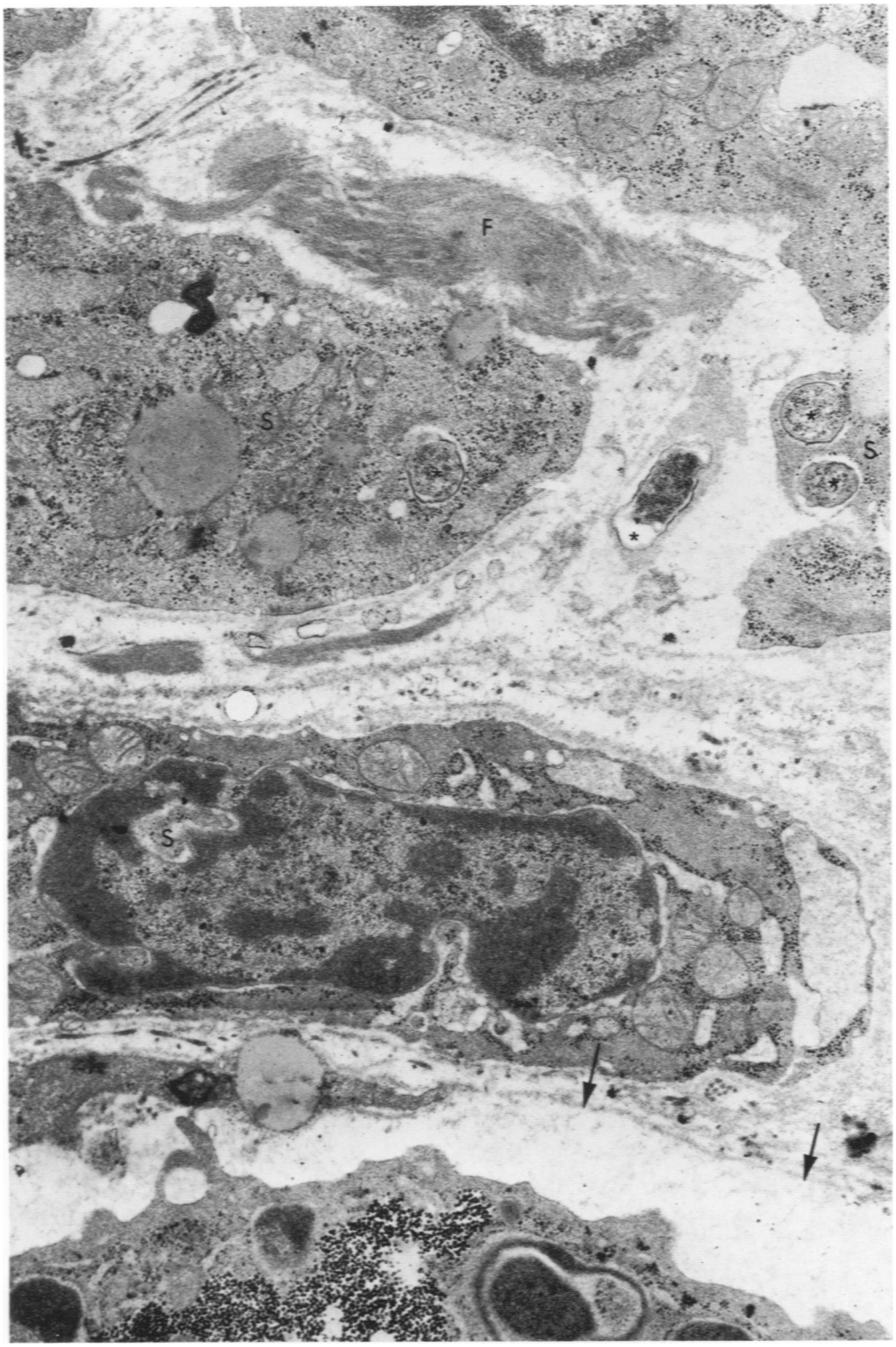

Figure 10—Epididymal vessel from guinea pig infected with *R. rickettsii* and maintained with saline treatment until sacrificed when moribund on Day 13. An endothelial cell (*arrows*) has dropped out from its position in the vessel wall. Rickettsiae (*) are present in smooth muscle cells (*S*) and extracellularly along with abundant fibrin (*F*) deposition. (× 20,300)

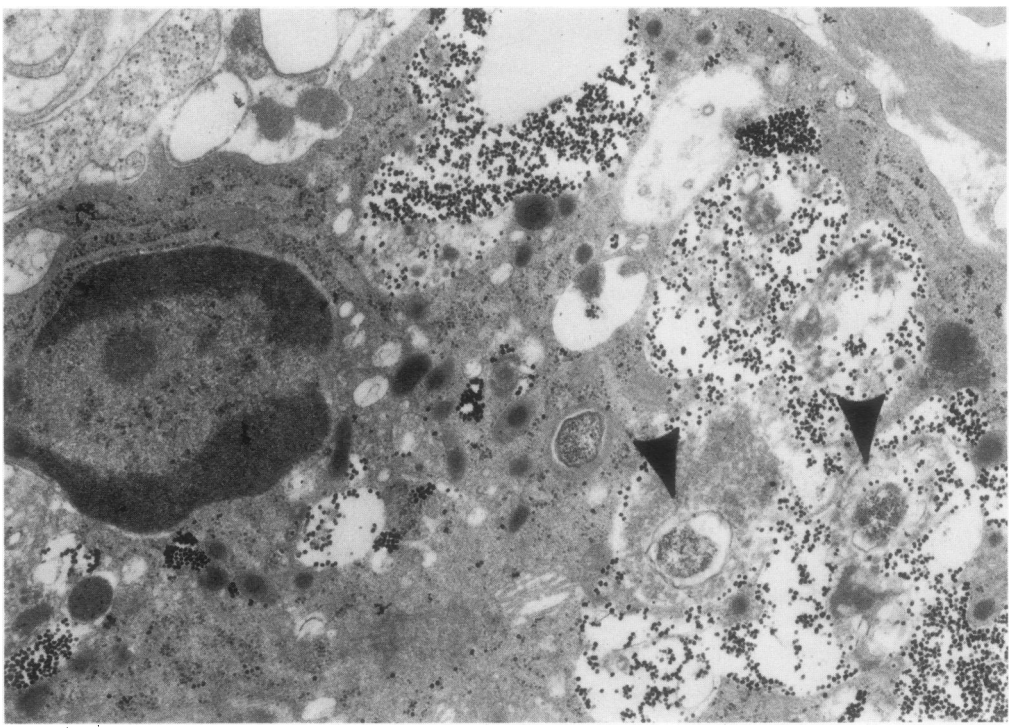

Figure 11—Epididymis from guinea pig infected with *R. rickettsii* and maintained with saline treatment until sacrificed when moribund on Day 11. Polymorphonuclear leukocytes with phagolysosomes containing rickettsiae (*arrows*) which vary in their structural integrity. (× 18,650)