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Ultrastructural Study of Ehrlichial Organisms in the Large Colons of Ponies Infected with Potomac Horse Fever

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Potomac horse fever is characterized by fever, anorexia, leukopenia, profuse watery diarrhea, dehydration, and high mortality. An ultrastructural investigation was made to search for any unusual microorganisms in the digestive system, lymphatic organs, and blood cells of ponies that had developed clinical signs after transfusion with whole blood from horses naturally infected with Potomac horse fever. A consistent finding was the presence of rickettsial organisms in the wall of the intestinal tract of these ponies. The organisms were found mostly in the wall of the large colon, but fewer organisms were found in the small colon, jejunum, and cecum. The organisms were also detected in cultured blood monocytes. In the intestinal wall, many microorganisms were intracytoplasmic in deep glandular epithelial cells and mast cells. Microorganisms were also found in macrophages migrating between glandular epithelial cells in the lamina propria and submucosa. The microorganisms were round, very pleomorphic, and surrounded by a host membrane. They contained fine strands of DNA and ribosomes and were surrounded by double bileaflet membranes. Their ultrastructure was very similar to that of the genus *Ehrlichia*, a member of the family *Rickettsiaceae*. The high frequency of detection of the organism in the wall of the intestinal tract, especially in the large colon, indicates the presence of organotrophism in this organism. Infected blood monocytes may be the vehicle for transmission between organs and between animals. The characteristic severe diarrhea may be induced by the organism directly by impairing epithelial cell functions or indirectly by perturbing infected macrophages and mast cells in the intestinal wall or by both.

Potomac horse fever (PHF) has been reported with apparently increasing frequency during the summer months of the last 7 years in the counties adjacent to the Potomac River in Maryland and Virginia (9). Reports of the disease have recently been received from other areas in the United States (J. E. Palmer, personal communication). The clinical signs of the disease include fever, anorexia, leukopenia, watery diarrhea, and dehydration (9). In 1983, 42 of 116 Maryland horses affected with PHF died or were euthanatized. Among the 32 cases reported in Virginia, there were 10 deaths. In Pennsylvania, there were 4 deaths in 25 cases, and in 1984 18 of 108 horses in the same region died.

Examinations for salmonellae have proved negative, and clostridial toxins do not appear to play a role in the etiology of the disease (2). From a recent epidemiological survey it was concluded that the disease is infectious, but not contagious (B. D. Perry, J. E. Palmer, J. B. Birch, R. A. Magnusson, D. Morris, and H. F. Troutt, Proc. Soc. Vet. Epidemiol. Prev. Med., 1984, p. 148–153. The isolation of a coronavirus-like agent from affected horses was reported (7), but this agent did not cause disease when it was inoculated into ponies (9). After reports that disease could be transmitted by the inoculation of whole blood from horses with early untreated PHF into susceptible horses (R. H. Whitlock, J. E. Palmer, C. E. Benson, H. M. Acland, A. L. Jenny, and M. Ristic, Proc. 27th Annu. Meet. Am. Assoc. Vet. Lab. Diagnosticians, 1984, p. 103–124), it was reported that recovered horses developed serum antibodies to *Ehrlichia sennetsu*, as detected by the fluorescent antibody technique (A. L. Jenny, Am. Assoc. Vet. Pract. Newsl. no. 2, p. 64–65, 1984). There was no seroconversion to *Ehrlichia equi*, but some slight seroconversion to *Ehrlichia canis* (the etiological agent of canine tropical pancytopenia) occurred (6). The clinical signs of PHF differ from equine ehrlichiosis caused by *E. equi* (4). Equine ehrlichiosis has a lower mortality rate and is not associated with diarrhea.

We had previously reported briefly on the first demonstration of microorganisms with an ultrastructure similar to that of the genus *Ehrlichia* in macrophages of the large colon of a pony infected by transfusion of whole blood from a horse with early untreated natural PHF (Y. Rikihisa, B. D. Perry,

TABLE 1. Identification of ehrlichiae in organs by electron microscopy

Organ	Detection of ehrlichiae in pony no.[a]:						
	1	2	3	5	7	8	13
Duodenum	/	/	/	/	/	−	−
Jejunum	+	+	+	/	/	/	/
Ileum	/	/	/	/	+	−	/
Cecum	−	/	−	−	++	++	+
Large colon	+++	+++	+++	++	++	++	+
Small colon	/	++	++	/	+	−	+
Stomach	−	−	−	/	−	−	−
Liver	−	−	−	/	/	/	/
Spleen	−	−	−	/	−	−	−
Lymph nodes							
Mesenteric	−	−	−	/	/	/	−
Ileal	/	/	/	/	/	−	/
Cecocolic	/	/	/	/	+	/	/
Cerebrum	−	/	−	/	/	/	/
Cerebellum	−	/	−	/	/	/	/

[a] +, Ehrlichiae detected; −, ehrlichiae not detected; /, Specimens not examined.

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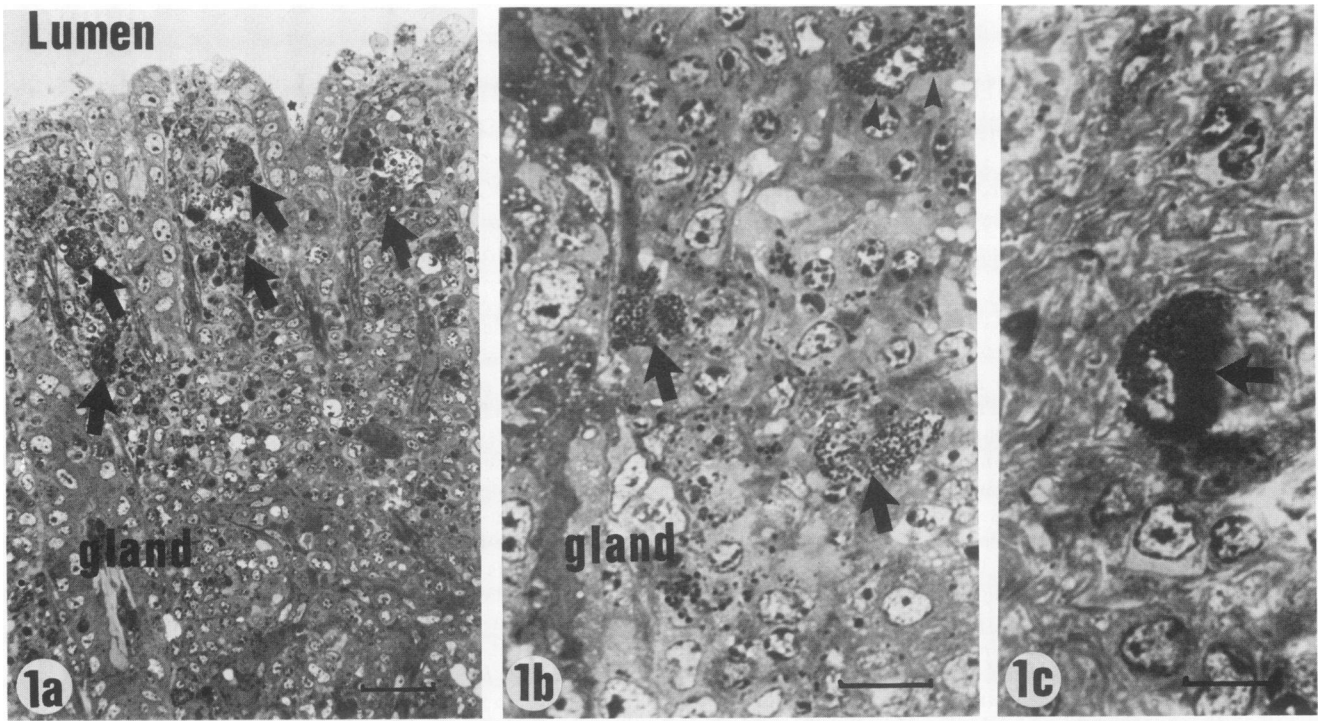

FIG. 1. (a) Thick vertical section through the wall of the Epon-embedded large colon of an infected pony. Infiltrations of mast cells, eosinophils, lymphocytes, and plasma cells are seen in the lamina propria. Note the focal necrotic regions (arrows) in the lamina propria. Toluidine blue stain. Bar, 20 μm. (b) Higher magnification of the deeper portion of the lamina propria of the large colon showing cells containing numerous dark stained microorganisms (arrows). Note most of the mast cell granules are larger (arrow heads) than the microorganisms. Toluidine blue stain. Bar, 10 μm. (c) Thick section of Epon-embedded large colon of an infected pony. A macrophage in the submucosa contains numerous intracytoplasmic microorganisms (arrow). Toluidine blue stain. Bar, 10 μm.

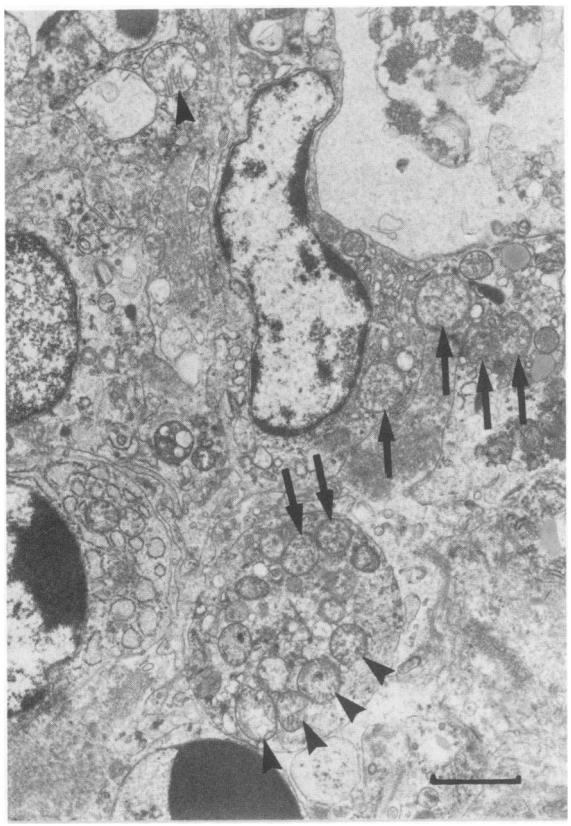

FIG. 2. Transmission electron micrograph of macrophages in the lamina propria of the large colon of an infected pony. Note numerous ehrlichial organisms (arrows) in the cytoplasm of macrophages. The connective tissue contains collagen bundles as well as much debris from disrupted cells. Also note swollen mitochondria with sparse cristae in the cytoplasm of macrophages and other cells (arrow heads). Bar, 1 μm.

and D. O. Cordes, Vet. Rec. Lett. **115**:390, 1984). This is the full report on this investigation. Later we isolated this organism in a human histiocyte cell line from PHF-infected pony leukocytes (Y. Rikihisa and B. D. Perry, Vet. Rec. Lett. **115**:554, 1984); the organisms were ultrastructurally identical to the microorganisms found in the infected colons. Inoculation of this organism to ponies resulted in clinical manifestations that were similar to those seen in the natural disease, and the organisms were reisolated from blood of inoculated ponies (6, 15). Although the organism was identified as the causative agent of PHF, its localization and detailed ultrastructure in the infected horse have not been reported. In this article we focus on a detailed description of the pleomorphic ultrastructure of the ehrlichial organisms and their host tissue and cell associations in the experimentally infected ponies.

MATERIALS AND METHODS

Infection of ponies. Two female ponies were each experimentally infected by the intravenous transfusion into the jugular vein of 350 ml of whole blood obtained from two

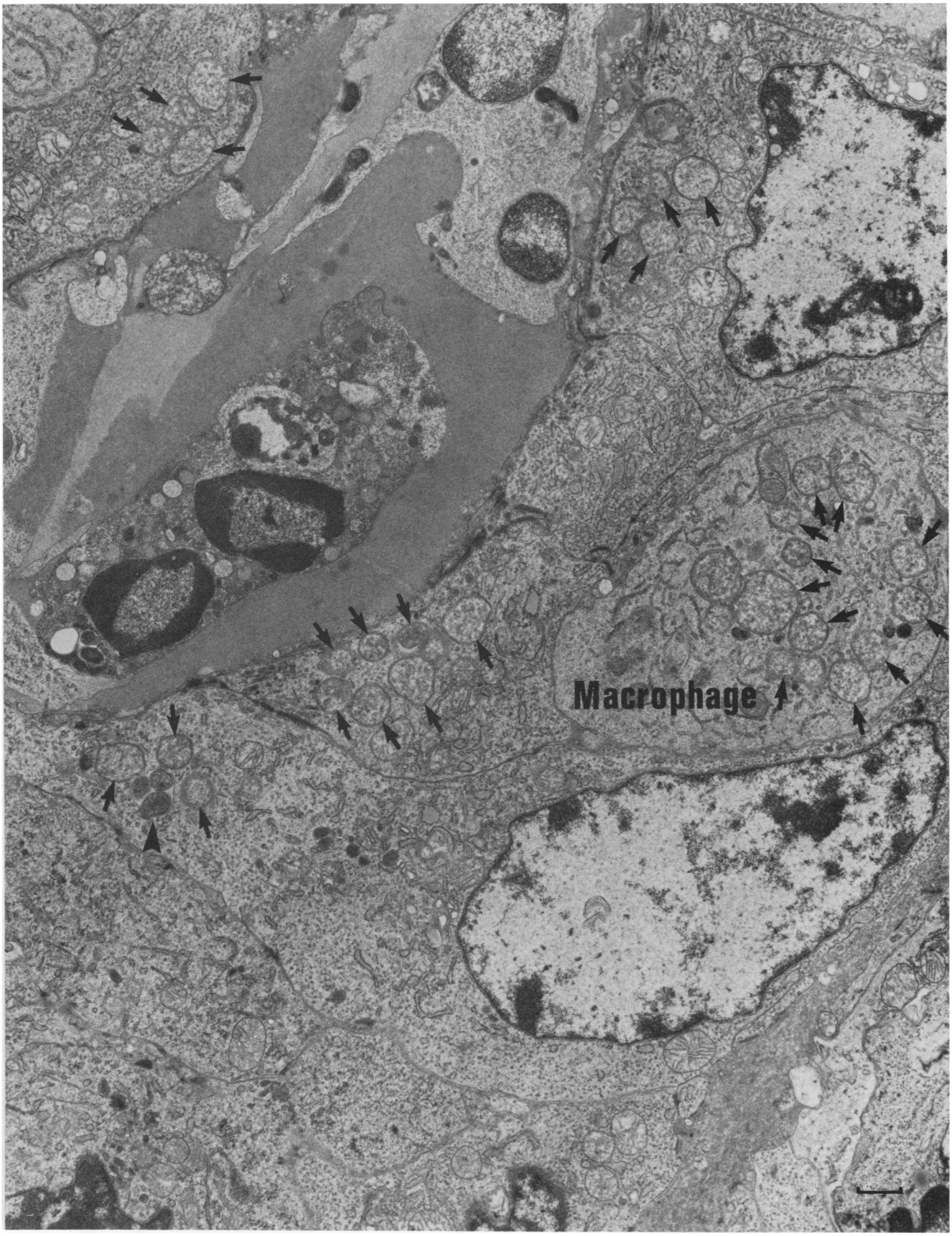

FIG. 3. Ehrlichial organisms (arrows) in the glandular epithelial cells. Most of them occur individually; some small organisms made clusters in a vacuole (arrow head). Note a migrating macrophage with numerous intracytoplasmic microorganisms. Bar, 1 μm.

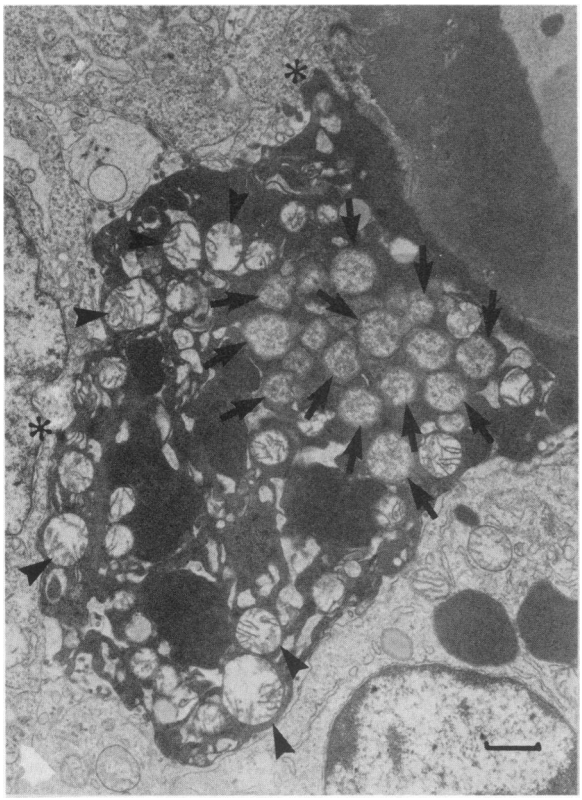

FIG. 4. Dying epithelial cell still maintaining its intercellular junction (*) contained numerous ehrlichiae (arrows). Note swollen mitochondria (arrow heads) Bar, 1 μm.

early untreated field cases of the disease in Maryland. After an incubation period of 9 to 14 days, the ponies were febrile (38 to 40°C) and had a lymphopenia (772 to 1,188 lymphocytes per μl), accompanied by depression and anorexia. Another group of 14 ponies was subsequently transfused from these two ponies. All ponies were seronegative to the antigens of *Babesia caballi*, *Babesia equi*, and equine infectious anemia virus (A. L. Jenny, personal communication). In all the ponies except one, clinical signs typical of PHF developed. Diarrhea developed in 88% of the experimentally infected ponies. The ponies were euthanatized by intravenous injection of T-61 (American Hoechst Corp., Somerville, N.J.) for necropsy 1 to 5 days after the onset of clinical signs. Three noninfected ponies were also euthanatized to examine any common microorganisms or viruses in their tissue and normal structure of their organs.

Blood leukocyte culture. Leukocyte fractions were prepared from blood (350-ml volume) obtained from ponies in the early febrile stage of the disease. Leukocytes were aseptically separated with Histopaque 1077 (Sigma Chemical Co., St. Louis, Mo.). Pony leukocytes were cultured in square flasks (25-cm^2 culture area) with RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% calf serum at 37°C in a humidified atmosphere of 5% CO_2–95% air. Seven days later, the monocyte monolayer was fixed for electron microscopic observation.

Electron microscopy. At necropsy, specimens (10 to 20 samples per organ per pony) of digestive and lymphatic organs were prepared for transmission electron microscopy as previously described (14). Briefly, specimens were cut into 3-mm cubes and fixed overnight at 4°C in a mixture of 2.5% paraformaldehyde, 5% glutaraldehyde, and 0.03%

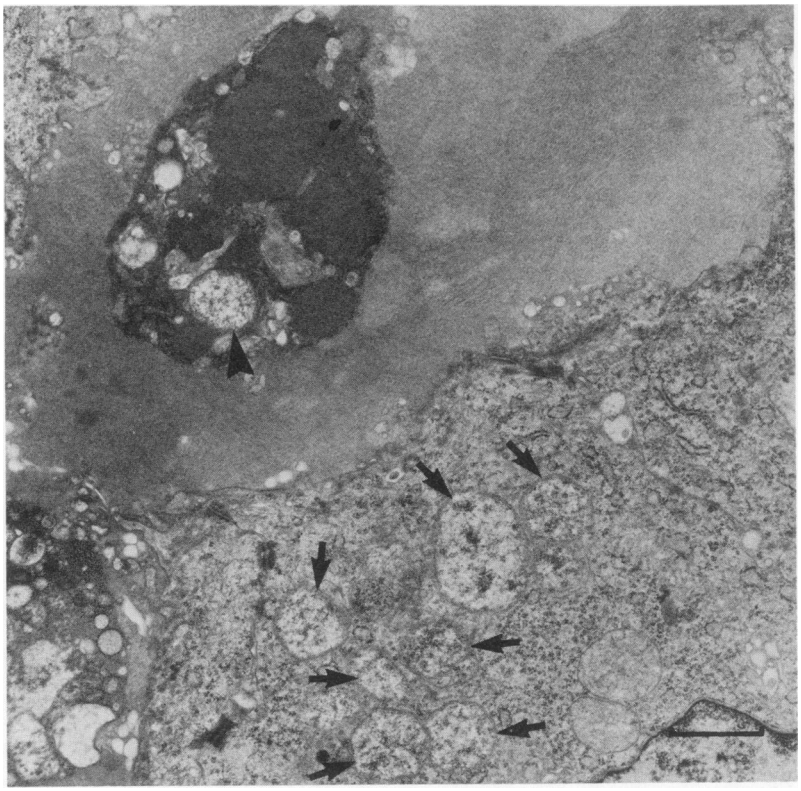

FIG. 5. Exfoliated cell in the glandular lumen carrying an ehrlichia (arrow head). Glandular epithelial cells also contain numerous ehrlichiae (arrows). Bar, 1 μm.

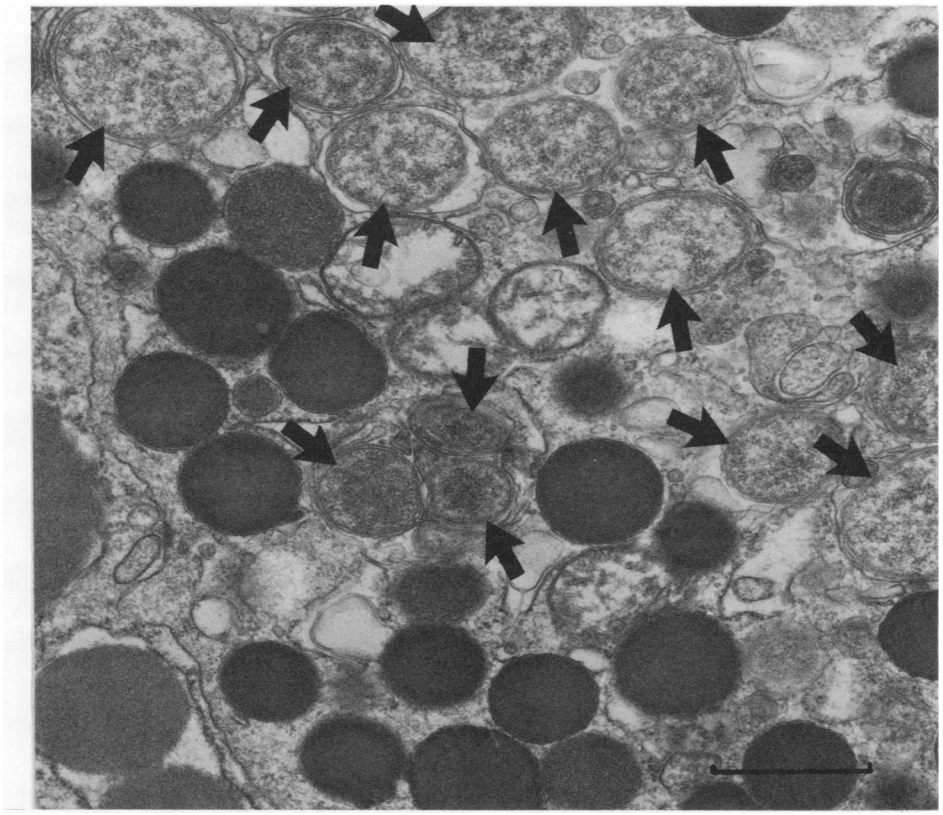

FIG. 6. Ehrlichial organisms (arrows) in the cytoplasm of a mast cell in the submucosa of the colon of an infected pony. All of the microorganisms were surrounded by the host cell membranes. Note that mast cell granules have not coalesced with ehrlichia-containing vacuoles. Bar, 1 μm.

trinitrophenol in 0.1 M cacodylate buffer (pH 7.4) and postfixed in 1% OsO_4 in 1.5% potassium ferrocyanide. After block staining in 1% uranyl acetate in maleate buffer (pH 5.2), tissues were dehydrated in a graded series of ethanols and propylene oxide and embedded in Poly/Bed 812 (Polysciences, Inc., Warrington, Pa.). Blood monocytes cultures were treated the same way. The cells were scraped off the bottom of the culture flask with a razor blade before being transferred to propylene oxide. Thin sections (60 to 90 nm) were cut, stained with uranyl acetate and lead citrate, and examined with a JEM 100 CXII electron microscope.

RESULTS

With electron microscopy, rickettsial microorganisms were consistently found in the wall of the large colon of the ponies that had developed clinical signs of PHF (Table 1), whereas they were absent in the intestinal organs of control ponies. They were also detected in the small colon, jejunum, ileum, cecum, and cecocolic lymph node. The rickettsial organisms were not detected in the walls of duodenums, gastric mucosa, or other organs examined (Table 1). The organisms were detected by electron microscopy in the small number of monocytes derived from pony blood only after they had been in culture for 7 days. No other microorganisms or viruses were found consistently in any organs examined.

Since the host cell association of the organism was identical throughout the intestinal tract and the largest numbers of organisms were detected in the walls of the large colons of the infected ponies, our present study illustrates the ultrastructure of this organism in that organ.

At low magnification, the intestinal wall where rickettsiae were identified by transmission electron microscopy was almost intact. The intestinal glands (crypts), lamina propria, submucosa, muscularis externa, and serosal layers were all present. In comparison to controls, infiltration of mast cells, eosinophils, lymphocytes, and plasma cells was more pronounced, and congestion of some small blood vessels in the lamina propria and submucosa was found.

With light microscopy it was very difficult to detect the rickettsial microorganisms in any of the tissues examined, unless the cells were heavily infected (Figs. 1b and c). The organisms were round and stained dark purple with toluidine blue and reddish-purple with Giemsa stain. They were generally smaller and less distinct than the mast cell granules. Eosinophils had easily distinguishable, much larger, refractile, greenish-blue granules with the toluidine blue stain.

Small, focal necrotic areas were observed in the lamina propria between the intestinal glands in some areas of the large colon of infected ponies (Fig. 1a). When these areas were observed by electron microscopy, macrophages containing numerous rickettsial organisms were found in the connective tissue containing much debris from disrupted cells (Fig. 2). The organisms were also found in the macrophages in lamina propria (Fig. 1b) and submucosa (Fig. 1c), which appeared intact. A large number of organisms were found in glandular epithelial cells (Fig. 3) and in the macrophages migrating among them (Fig. 3). Exfoliating epithelial cells, still maintaining some of their intercellular junctions, also contained numerous organisms (Fig. 4). There were some degenerating cells in the lumen of the intestinal glands

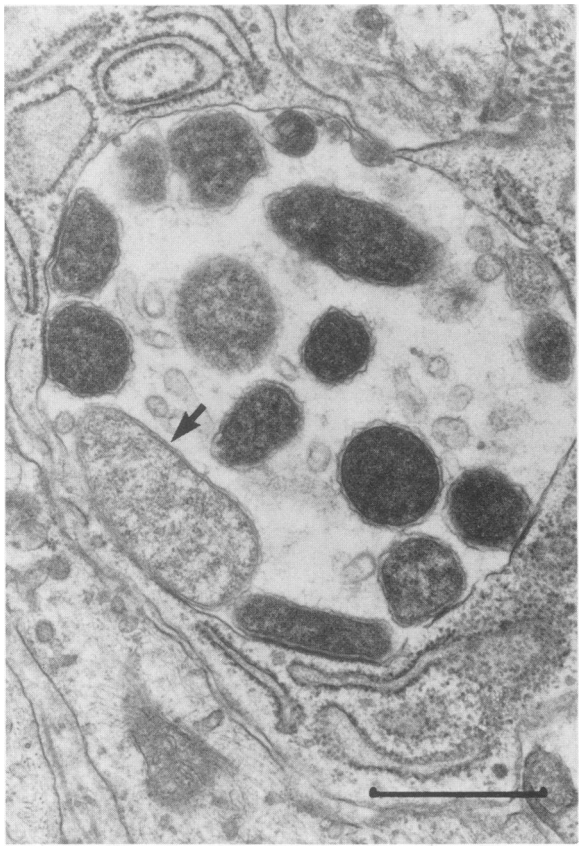

FIG. 7. Dense and smaller forms of organisms and a lighter and larger form are found in the same vacuole. The larger one (arrow) occurs peripherally and is apparently tightly associated with the host membrane. It would probably later pinch off from the rest of the vacuole, leaving remaining smaller and denser organisms. Bar, 1 μm.

that contained rickettsiae (Fig. 5), indicating that microorganisms were actually shed into the lumen. The same microorganisms were also found in the cytoplasm of mast cells in the lamina propria and submucosa (Fig. 6). The mast cell granules did not coalesce with organism-containing membrane vesicles (Fig. 6). The organism was rarely found in plasma cells. The organisms were limited to these four types of cells. Goblet cells and endocrine cells in the glandular epithelium endothelial cells, eosinophils, neutrophils, erythrocytes, smooth muscle cells, fibroblasts, and mesothelial cells were all negative for their presence.

The microorganisms were round and very pleomorphic, and at least two forms were discernible. There were also intermediate forms. Small (0.2 to 0.4 μm in diameter) electron-dense forms (Fig. 3, 7, and 8), including organisms undergoing binary fission (Fig. 9), were found in loose host membrane vacuoles (Fig. 3, 7, 8, and 11). Most of the larger (0.6 to 1.5 μm in diameter) and less electron-dense organisms were individually enveloped by very tight host membrane (Fig. 3, 4, 5, 6, and 10). These larger forms were difficult to identify even under the electron microscope because of their similar density to the host cell cytoplasm. Although the two forms were mostly in separate host vacuoles, occasionally smaller electron-dense organisms and peripherally located electron-lucent organisms were found in the same vacuole (Fig. 7), suggesting that the host membrane surrounding the larger forms was being pinched off from the main vacuole. Some vacuoles contained small, single membrane vesicles apparently derived from the outer membrane of the organism (Fig. 8). Some small, double membrane vesicles (0.05 to 0.1 μm in diameter) with electron-dense cytoplasm were also found (Fig. 11), suggesting the occurrence of unequal binary fission.

The organisms contained fine strands of DNA and ribosomes (Fig. 10) and were surrounded by double bileaflet membranes. Smaller, dark forms had an extensive fuzzy coating on the outer membrane (Fig. 11). The thickening of the outer or inner leaflet of either membrane, suggesting the presence of the peptidoglycan such as that reported in the genus *Rickettsia* (18), was not found in either form of the organism.

Despite the large number of microorganisms they contained, most host cells were morphologically intact, except for the presence of swollen mitochondria (Fig. 2 and 4). Similar swollen mitochondria were also seen in circulating monocytes of the infected ponies.

DISCUSSION

Identical rickettsial organisms were consistently found in the ponies transfused with blood from horses naturally infected with PHF, indicating that this organism was transmitted from horses to ponies via the blood. No other microorganisms or virus were consistently identified in these ponies.

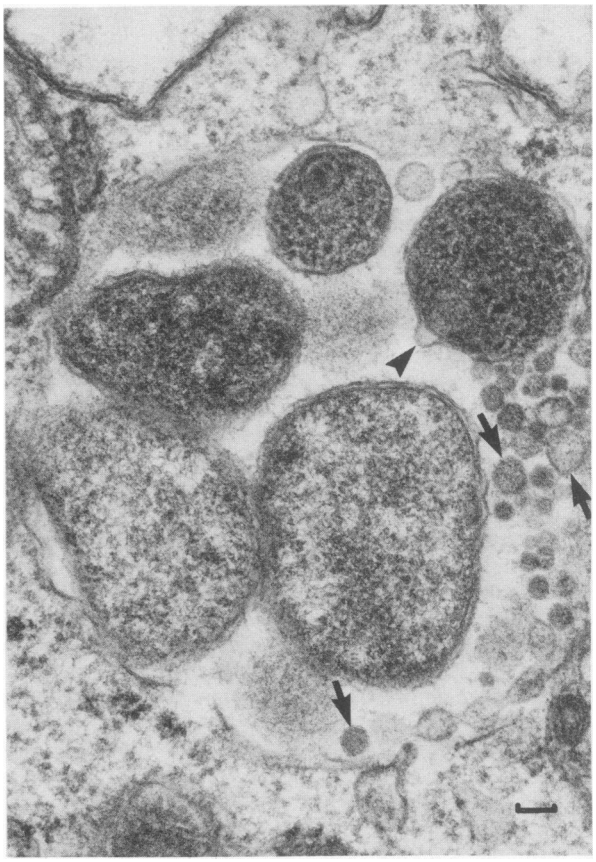

FIG. 8. Some vacuoles contain, in addition to intact ehrlichiae, many membrane vesicles (arrows); apparently the outer membrane vesicles pinched off (arrow head). Bar, 0.1 μm.

These rickettsial organisms were found throughout the intestinal tract and were highly concentrated in the wall of the large colon. Organisms were intracytoplasmic and not found outside of the host cell, supporting our tissue culture data indicating that these are obligate intracellular parasites (Rikihisa and Perry, Vet. Rec. Lett. **115**:554, 1984). The organisms were round and host membrane-bound, thus different from the genus *Rickettsia*. Although a small proportion of affected and unaffected horses in Maryland have been shown to be seropositive to *Rickettsia rickettsii* (Perry and Farhang-Azad, unpublished data), this was apparently independent of previous exposure to PHF. This result also suggests an absence of cross-protective immunity between *R. rickettsii* and the PHF agent. The organism was ultrastructurally rather similar to the genus *Ehrlichia*, thereby supporting the serological study of Jenny (Am. Assoc. Vet. Pract. Newsl. no. 2, p. 64–65, 1984.), in which infected horses were found to develop serum antibodies against *E. sennetsu*. With respect to host cell specificity, however, the PHF agent is different from any of the known ehrlichial organisms. *E. canis* (13) and *E. sennetsu* (5) are found in monocytes or macrophages, and *E. equi* is found in granulocytes (4). Other members of the tribe *Ehrlichieae*, such as organisms of the genus *Cowdria*, grow preferentially in endothelial cells (19), and organisms of the genus *Neorickettsia* grow in reticulocytes in the lymph nodes of dogs (1). In fact, none of the rickettsiae found so far prefers to grow in intestinal epithelial cells of mammals, although many of them grow in the gut of their blood-sucking arthropod vectors (8, 16, 17).

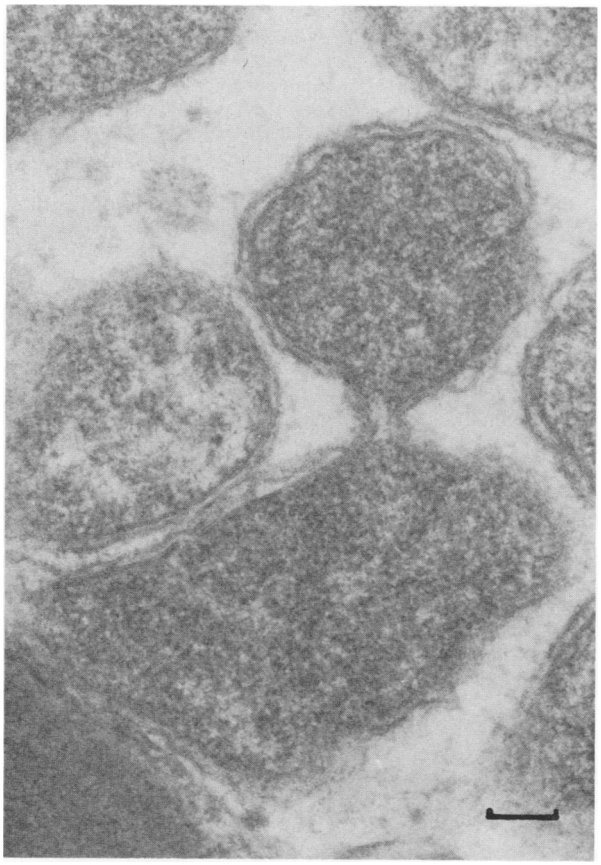

FIG. 9. Ehrlichiae in binary fission in the cytoplasm of glandular epithelial cells of the colon. Bar, 0.1 μm.

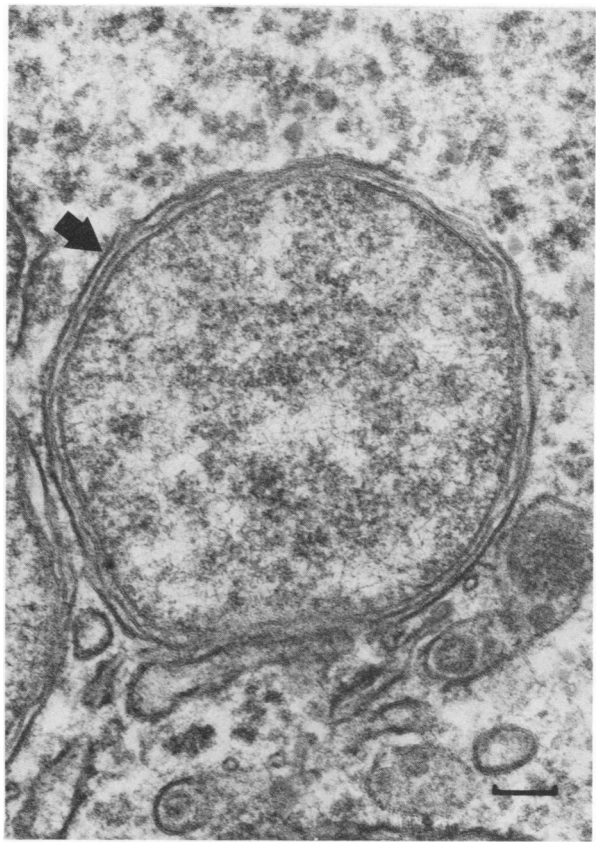

FIG. 10. Larger ehrlichial organism individually enveloped with a tight host membrane (arrow). Note its two unit membranes, clear DNA filaments, and ribosomes. Bar, 0.1 μm.

The PHF agent was very pleomorphic, suggesting a development from electron-dense forms to larger electron-lucent forms similar to *Chlamydia* spp. (12). However, unlike *Chlamydia* spp., binary fission was found in the electron-dense forms. The numerous aberrant forms, such as outer membrane vesicles or minicell-like structures (3), may not play a direct role in the life cycle of the PHF agent, but may have an effect on the host immune response once they are released extracellularly.

The most characteristic clinical sign of this disease is a progressive fluid accumulation in the large intestine, followed by a sometimes "explosive" diarrhea, possibly due to impaired fluid adsorption in the intestinal wall. In *Campylobacter jejuni* and *Shigella* spp. infections, mucosal damage and inflammation account for the ensuing diarrhea (10); however, such extensive damage was not a consistent finding in PHF. The mucosa was relatively intact as in cholera (11), suggesting that interference with epithelial cell function may be caused biochemically by the organisms multiplying in epithelial cells, macrophages, and mast cells.

In the present study, the sloughed epithelial cells containing ehrlichiae observed in the lumen of the intestinal glands suggest the possibility of transmission through contamination of grasses or soil with feces containing exfoliated epithelial cells bearing live organisms. Preliminary experiments involving oral administration of fecal material from affected horses (J. E. Palmer, personal communication) in-

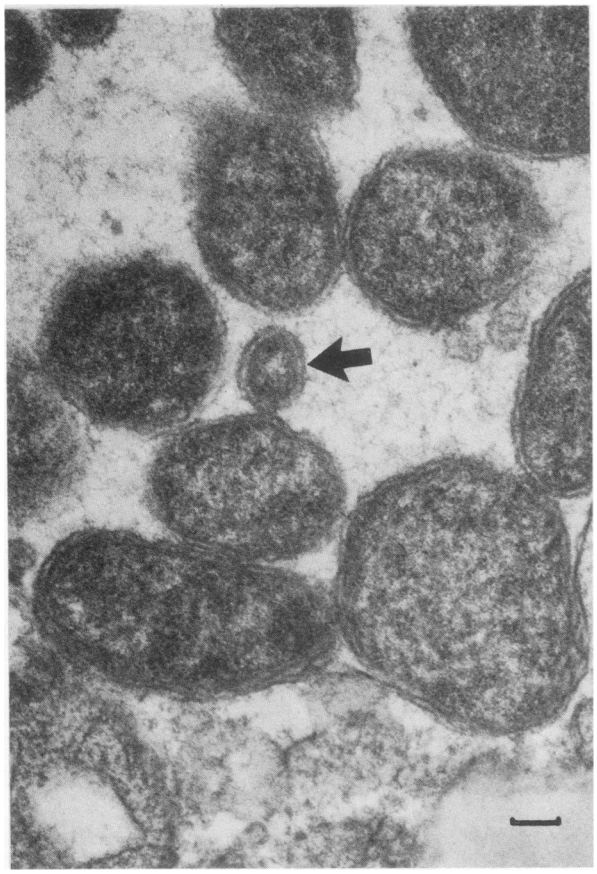

FIG. 11. Vacuole containing minicell-like structures (arrow) as well as ehrlichial organisms. Note extensive fuzzy coating extending from outer membrane. Bar, 0.1 μm.

dicate, however, that fecal material from clinically affected horses is not likely infective to susceptible horses. The seasonal nature of the disease and its restricted geographical distribution suggest that it is arthropod borne. This would imply that the multiplication of the organism in the epithelial cells in the intestinal wall plays a primary role in the pathogenesis of the disease, but is not of significance in its transmission. Although the organisms were fewer in number, circulating blood monocytes may be the vehicle for transmission of organisms between animals.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Brown, J. L., D. L. Huxsoll, M. Ristic, and P. K. Hildebrandt.** 1972. *In vitro* cultivation of *Neorickettsia helminthoeca*, the causative agent of salmon poisoning disease. Am. J. Vet. Res. **33**:1695–1700.
2. **Ehrich, M., B. D. Perry, H. F. Troutt, R. W. Dellers, and R. A. Magnusson.** 1984. Acute diarrhea in horses of the Potomac River area: examination for clostridial toxins. J. Am. Vet. Med. Assoc. **185**:433–435.
3. **Frazer, A. C., and R. Curtiss III.** 1975. Production, properties, and utility of bacterial minicells. Curr. Top. Microbiol. Immunol. **69**:1–84.
4. **Gribble, D. H.** 1969. Equine ehrlichiosis. J. Am. Vet. Med. Assoc. **155**:462–469.
5. **Hoilien, C. A., M. Ristic, D. L. Huxsoll, and G. Rapmund.** 1982. *Rickettsia sennetsu* in human blood monocyte cultures: similarities to the growth cycle of *Ehrlichia canis*. Infect. Immun. **35**:314–319.
6. **Holland, C. J., M. Ristic, A. I. Cole, P. Johnson, G. Baker, and T. Goetz.** 1985. Isolation, experimental transmission, and characterization of causative agent of Potomac horse fever. Science **227**:522–524.
7. **Huang, J. C. M., S. L. Wright, and W. D. Shipley.** 1983. Isolation of coronavirus-like agent from horses suffering from acute equine diarrhea syndrome. Vet. Rec. **113**:262–263.
8. **Ito, S., J. W. Vinson, and T. J. McGuire, Jr.** 1975. Murine typhus rickettsiae in the oriental rat flea. Ann. N.Y. Acad. Sci. **266**:35–60.
9. **Knowles, R. C., D. W. Anderson, W. D. Shipley, R. H. Whitlock, B. D. Perry, and J. D. Davidson.** 1983. Acute equine diarrhea syndrome (AEDS): a preliminary report. Proc. Am. Assoc. Equine Pract. **29**:353–357.
10. **Mata, L.** 1978. Diarrheal diseases; a leading world health problem, p. 1–14. 43rd Nobel Symposium, Stockholm, Sweden. S. Karger, New York.
11. **Mims, C. A.** 1982. The pathogenesis of infectious disease. Academic Press, Inc., New York.
12. **Moulder, J. W.** 1984. Looking at chlamydiae without looking at their hosts. Am. Soc. Microbiol. News **50**:353–362.
13. **Nyindo, M. B. A., M. Ristic, D. L. Huxsoll, and A. R. Smith.** 1975. Tropical canine pancytopenia: *in vitro* cultivation of the causative agent *Ehrlichia canis*. Am. J. Vet. Res. **32**:1651–1658.
14. **Rikihisa, Y., and S. Ito.** 1979. Intracellular localization of *Rickettisa tsutsugamushi* in polymorphonuclear leukocytes. J. Exp. Med. **150**:703–708.
15. **Rikihisa, Y., B. D. Perry.** 1985. Causative ehrlichial organisms in Potomac horse fever. Infect. Immun. **49**:513–517.
16. **Shkolnik, L. Y., B. G. Zatulovsky, and N. M. Shestopalova.** 1966. An electron microscope study of ultrathin sections from infected louse guts and chick embryo yolk saks. Acta Virol. **10**:260–265.
17. **Silverman, D. J., J. L. Boese, and C. L. Wisseman, Jr.** 1974. Ultrastructural studies of *Rickettsia prowazekii* from louse midgut cells to feces: search for "dormant" forms. Infect. Immun. **10**:257–263.
18. **Silverman, D. J., and C. L. Wisseman, Jr.** 1978. Comparative ultrastructural study on the cell envelopes of *Rickettsia prowazekii*, *Rickettsia rickettsii*, and *Rickettsia tsutsugamushi*. Infect. Immun. **21**:1020–1023.
19. **Uilenberg, G.** 1981. Heartwater disease, p. 345–360. *In* M. Ristic and I. McIntyre (ed.), Disease of cattle in the tropics. Martinus Nijhoff Publishers, The Hague, Holland.