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15. We are continuing efforts to improve the precision of deuterium measurements of fluid inclusions, beginning to examine the ^{18}O content of the vein calcite, and expanding petrologic studies in an attempt to understand the nature of the vein laminae.
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10 August 1984; accepted 11 October 1984

Isolation, Experimental Transmission, and Characterization of Causative Agent of Potomac Horse Fever

Abstract. *Potomac horse fever, a disease characterized by fever, anorexia, leukopenia, and occasional diarrhea, is fatal in approximately 30 percent of affected animals. The seasonal occurrence of the disease (June to October) and evidence of antibodies to the rickettsia* Ehrlichia sennetsu *in the serum of convalescing horses suggested that a related rickettsia might be the causative agent. Such an agent was isolated in cultured blood monocytes from an experimentally infected pony. This intracytoplasmic organism was adapted to growth in primary cultures of canine blood monocytes. A healthy pony inoculated with these infected monocytes also developed the disease. The organism was reisolated from this animal which, at autopsy, had pathological manifestations typical of Potomac horse fever. Cross serologic reactions between the newly isolated agent and antisera to 15 rickettsiae revealed that it is related to certain members of the genus* Ehrlichia, *particularly to* Ehrlichia sennetsu. *Since the disease occurs in other parts of the United States as well as in the vicinity of the Potomac River, and since it has also been reported in Europe, the name equine monocytic ehrlichiosis is proposed as being more descriptive.*

Potomac horse fever, a disease characterized by fever, anorexia, leukopenia, and occasional diarrhea, is fatal in approximately 30 percent of affected animals. The disease was first reported in the vicinity of the Potomac River in the state of Maryland in 1979 (*1*). Since then, the disease has become well established in the eastern part of the United States and has been observed sporadically in other parts of the country and abroad (*2*).

Over the last 5 years, many state and government laboratories have been investigating the cause of the disease. A number of viruses and bacterial agents and their by-products have been demonstrated in the blood and tissues of affected animals, but none of these have been proved to be etiologically related to the disease (*3*).

This study was prompted by three research leads regarding the possible nature of the causative agent. First, the disease was found to have a seasonal occurrence, with most cases being observed from June through October. Second, it was demonstrated that the infection could be experimentally transmitted by blood transfusion from acutely infected to susceptible horses (*4*). Finally, studies in our laboratory revealed antibodies in the serum of convalescing animals that reacted with *Ehrlichia sennetsu* in the indirect fluorescent antibody (IFA) test (*5*). *Ehrlichia sennetsu* is the causative agent of human sennetsu rickettsiosis, a disease that clinically resembles infectious mononucleosis and is prevalent in Japan and other regions of Southeast Asia (*6*). The most distinguishing characteristic of members of the genus *Ehrlichia* is their development within a membrane-lined cytoplasmic vacuole of leucocytes. The prototype for this genus, *E. canis*, is the etiologic agent of canine ehrlichiosis, a worldwide and frequently fatal disease of dogs. On the basis of these findings we directed our efforts toward isolation of the apparent causative rickettsia from the blood of experimentally infected horses and ponies.

A small volume (50 ml) of whole citrated blood from an experimentally infected animal during the acute stage of the disease was inoculated intravenously into a susceptible pony (*7*). The inoculated animal was monitored daily for evidence of clinical and hematological abnormalities. The animal developed a fever (104°F; 40°C) on day 13 after inoculation that was followed by anorexia, general depression, and diarrhea. Beginning on day 0, blood monocyte cultures were prepared from the inoculated animal at 72-hour intervals according to a method developed in our laboratory for the isolation of *E. canis* (*8*). The cells were propagated in 25-cm^2 tissue culture flasks with Medium 199 supplemented with 1 percent L-glutamine and 20 percent heat-inactivated normal horse serum. The cultures were maintained at 37°C in air, and were monitored by the

Table 1. Serological cross reactivities among the Potomac horse fever agent (PHFA), *Ehrlichia sennetsu, E. canis*, and *E. equi* as determined by the IFA test. N, no reaction at a serum dilution of 1:10.

Source of serum	Samples (No.)	Average titer			
		PHFA	*E. sennetsu*	*E. canis*	*E. equi*
PHF (equine)	5	1:160	1:20	N	N
experimental infection	2	1:320	1:40	1:10	N
	4	1:1280	1:80	1:10	N
	6	1:2560	1:160	1:10	N
	2	1:2560	1:320	1:20	N
PHF (equine) natural	8	1:160	1:20	N	N
infection	6	1:640	1:40	1:10	N
	3	1:1280	1:80	1:20	N
	2	1:2560	1:160	1:10	N
	3	1:2560	1:320	1:20	N
	2	1:5120	1:640	1:20	N
E. sennetsu (canine) experimental infection	3	1:40	1:640	1:80	1:10
E. canis (canine) experimental infection	5	1:10	1:80	1:640	1:80
E. equi (equine) experimental infection	3	N	1:20	1:80	1:640
Normal horse serum	10	N	N	N	N
Normal canine serum	10	N	N	N	N

Giemsa and the IFA methods for evidence of the causative agent (*9*).

Organisms closely resembling rickettsiae were first observed in the cytoplasm of cultured monocytes that were obtained from the blood of the pony on day 9 after inoculation. These singly occurring organisms were in evidence after 5 days of incubation in a relatively small percentage of monocytes (<5 percent). At this stage of incubation the monocytes appeared fragile with a moderate lytic effect becoming apparent. In an attempt to ensure further cultivation of the organism, and because of our experience in the cultivation of *E. canis* and *E. sennetsu* in primary blood monocyte cultures (*8, 10*), we transferred infectious material from the equine monocyte cultures to primary cultures of canine monocytes prepared from normal dogs (*11*).

Sequential microscopic examination of the inoculated canine monocyte cultures revealed evidence of a successful transfer of the organism. Using the Giemsa staining method, we observed that approximately 6 percent of the cells contained variable numbers of the agent by day 2 after transfer. Thereafter, there was a progressive increase in the number of infected monocytes reaching a maximum of 96 percent on day 8. At this time, numerous cells containing organisms in the form of loosely packed clusters were observed (Fig. 1). Many of these cells also contained inclusion bodies resembling those of *E. canis* and *E. sennetsu*. These inclusion bodies were pleomorphic with individual organisms clearly differentiated. In many instances the organisms appeared to be surrounded by a distinct vacuolar membrane. Disintegration of heavily infected monocytes with subsequent release of the organisms into the medium was observed at this late growth stage. The extracellular organisms remained in a nest-like arrangement held together by disrupted cell stroma. The organism has been maintained through a series of passages in primary cultures of canine blood monocytes. Uninoculated control cultures of monocytes from the same dog remained free of artifacts resembling these organisms.

Electron microscopic examination of infected canine monocytes revealed that singly occurring organisms and the inclusion bodies were surrounded by a distinct cytoplasmic vacuolar membrane (Fig. 2, A and B). The outer membrane of the organism appeared highly rippled, resembling a zipperlike arrangement (Fig. 2C). The organisms were pleomorphic, ranging in shape from round to oval and sausagelike (Fig. 2D); their internal structures consisted of highly electron dense material that was not clearly differentiated.

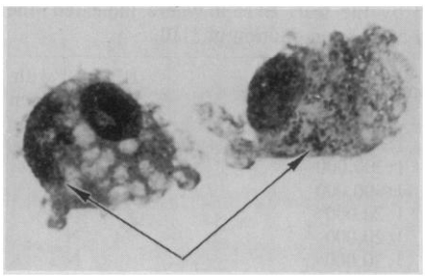

Fig. 1. Newly isolated causative agent of equine monocytic ehrlichiosis (Potomac horse fever) in the cytoplasm of cultured canine blood monocytes (arrows). This agent was identified as a rickettsia of the genus *Ehrlichia* (Giemsa, ×1500).

The specificity of the organism was ascertained by means of the IFA test (*9*). We used serial twofold dilutions of sera from horses that had recovered from Potomac horse fever. Serum samples from uninfected horses served as negative controls. Serum from the horses known to be infected (experimentally and naturally) reacted strongly with the newly isolated organism. There was less reaction with *E. sennetsu*, some with *E. canis*, and no reaction with *E. equi* antigens. The negative control sera showed no reaction with any of these antigens (Table 1). Cross reactions between the newly isolated agent and antisera to *E. sennetsu* and *E. canis* showed that these three organisms are antigenically related (Table 1). We then used the IFA test to examine antisera to 12 major rickettsiae for their reactivity with the new agent (*12*). None of these antisera reacted at a 1:10 dilution, thereby confirming the new agent's close relation to members of the genus *Ehrlichia* (Table 2).

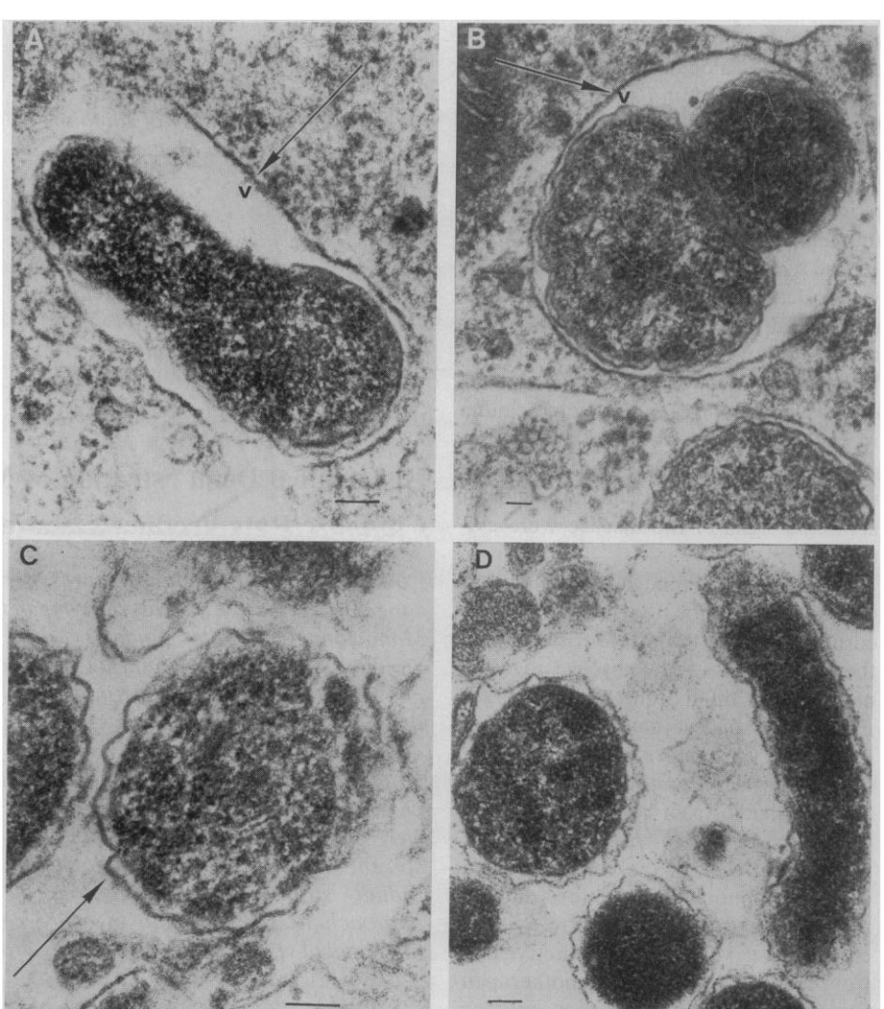

Fig. 2. Ultrastructure of the culture-derived causative agent of equine monocytic ehrlichiosis (Potomac horse fever). Single organisms and inclusion bodies are surrounded by a distinct cytoplasmic vacuolar membrane (see arrows in A and B). Note the highly rippled outer membrane of the organism (see arrow in C). The organisms appear pleomorphic, ranging from round to oval and sausagelike (D). Scale bars, 0.1 μm.

Table 2. The absence of cross reactivity between the Potomac horse fever agent (PHFA) and specific antisera to 12 rickettsiae as determined by the test. Except where indicated, the antisera were produced in rabbits. N, no reaction at a serum dilution of 1:10.

Antiserum to	Homologous titer*	IFA test with PHFA antigen
Rickettsia tsutsugamushi (Kato)	1:300,000	N
R. tsutsugamushi (Karp)	1:300,000	N
R. tsutsugamushi (Gilliam)	1:300,000	N
R. prowazeki	1:20,000	N
R. typhi	1:20,000	N
R. canada	1:30,000	N
R. conorii	1:30,000	N
R. akari	1:30,000	N
R. rickettsii†	1:8,192‡	N
Rochalimaea quintana	1:300,000	N
Rochalimaea vinsonii	1:300,000	N
Coxiella burnetti†	1:512†	N

*Except when indicated, the titer was determined by an enzyme immunoassay. †Antiserum obtained from naturally infected humans. ‡Titer determined by microagglutination.

To obtain conclusive evidence that the newly isolated organism is the causative agent of Potomac horse fever, we inoculated a susceptible pony with culture-derived organisms obtained from the third consecutive subpassage in primary canine monocyte cultures (*13*). The animal developed a fever (102° to 104.2°F) on day 7 after inoculation and by day 12 became depressed and anorexic. At this time diarrhea and slight edema on the distal portion of the hind legs were present. By using the methods described above we reisolated the organism from this animal in cultures initiated on days 10 and 12 after inoculation. The animal was killed on day 16 after inoculation and an autopsy revealed pathological manifestations consistent with Potomac horse fever (*14*). When a similar number of cells from control, noninfected canine monocyte cultures from the same donor dog were inoculated into another susceptible pony, no clinical, hematologic, or serologic signs of the disease were evident.

The name Potomac horse fever was chosen to describe the location of the earliest cases of the disease. In view of the occurrence of the disease in other parts of the United States and possibly in Europe, we suggest that the name equine monocytic ehrlichiosis would be more descriptive. This name would be clearly distinguishable from equine ehrlichiosis caused by *E. equi* (*6, 15*).

The discovery of the causative agent of equine monocytic ehrlichiosis and culture of the organism in vitro will facilitate the development of effective control measures including chemotherapeutics and, eventually, immunoprophylactics. It may now be possible to use serological tests to detect subclinical and overt forms of the disease and to determine its true geographic distribution. Since the causative organism is antigenically related to *E. sennetsu*, a human pathogen that has not, thus far, been demonstrated to occur in the western world (*6*), it may be necessary to examine the public health importance of the new agent.

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4. The agent described here has been maintained by serial blood transfusions from acutely infected to susceptible ponies at National Veterinary Services Laboratory (NVSL), Ames, Iowa.
5. Serum samples obtained from horses before and after they were experimentally infected with the Potomac horse fever agent as well as samples from naturally infected animals were examined in our laboratory for the presence of antibodies against *E. equi*, *E. canis*, and *E. sennetsu* by means of the IFA test.
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11. Volumes (1 ml) of supernatant medium from four infected equine monocyte cultures were transferred to primary canine monocyte cultures. The cultures were incubated for 1 hour at 37°C at which time 4 ml of fresh culture medium containing 20 percent normal dog serum was added to each flask.
12. Antisera against 12 rickettsiae were kindly provided by E. Weiss, U.S. Naval Medical Research Institute, and W. Burgdorfer, NIH, Rocky Mountain Laboratory.
13. A suspension of approximately 2×10^4 infected canine monocytes was centrifuged at 400*g*. The supernatant fluid was removed. The cell pellet was resuspended in 4 ml of sterile Hanks balanced salt solution (HBSS-Gibco). The cell suspension was then injected intravenously into a susceptible pony.
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16. We thank J. Dawson, I. Abeygunawardena and M. Khondowe for expert technical assistance.

15 October 1984; accepted 19 December 1984

Histological Demonstration of Mosaicism in a Series of Chimeric Rats Produced Between Congenic Strains

Abstract. *Experimental chimeras were produced by aggregating morulae from congenic strains of PVG rats differing in the major histocompatibility complex (RT1). Monoclonal antibodies against variant class I antigens of the two strains were directly conjugated to iodine-125 and applied to tissue sections. Autoradiograms allowed examination of most internal tissues. The proportion of PVG-*RT1^a *cells in the erythrocyte populations of the chimeras varied from 8 to 70 percent, as determined with fluorescence-activated flow cytometry. Digital analysis of autoradiograms demonstrated that the contribution of PVG-*RT1^a *cells to the livers of the chimeras ranged from 34 to 86 percent. Patches of cells of each genotype in the liver were geometrically complex, with large variations in size. The thymus, but not the spleen, showed evidence of oligoclonal development. The adrenal cortex revealed a radially striped pattern, suggestive of clonal expansion of stem cells. With this approach it is possible to measure cell distribution in chimeras through direct histological visualization, which may prove useful in the study of rat organogenesis.*

Chimeric (allophenic) animals have been produced experimentally in a number of mammalian species. Such animals are prepared by amalgamating two or more preimplantation embryos in vitro and transferring the chimeric embryos to surrogate mothers for intrauterine development (*1*). The adult tissues of these animals are mosaics composed of the progeny of the two or more cell lineages present in the original embryos. Analysis of the mosaic tissues has been used to