Adaptation of *Ehrlichia sennetsu* to Canine Blood Monocytes: Preliminary Structural and Serological Studies With Cell Culture-Derived *Ehrlichia sennetsu*

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*Ehrlichia sennetsu*, the causative agent of human sennetsu rickettsiosis, was successfully propagated in primary canine blood monocyte cultures. The growth cycle of this organism appears to be similar to that of *Ehrlichia canis*. The antigen derived from our *E. sennetsu* cultures was used to develop an indirect fluorescent antibody test for detection and titration of serum antibodies to the organism. Using this test system, we found that five human serum samples obtained from patients clinically diagnosed as having sennetsu rickettsiosis were positive for anti-*E. sennetsu* antibodies. In addition, 29% of the serum samples obtained from 200 patients having a fever of unknown origin and residing in various regions of Malaysia were also serologically positive.

All sera from apparently healthy individuals were negative in the test. Dogs inoculated with cell culture-adapted *E. sennetsu* developed a specific antibody titer to *E. sennetsu*, and the organism was subsequently isolated from their blood. These animals showed no clinical evidence of disease. The possibility of a higher prevalence of human sennetsu rickettsiosis in Southeast Asia and the potential usefulness of the canine model for studies of human sennetsu rickettsiosis are discussed.

*Ehrlichia sennetsu* is the causative agent of human sennetsu rickettsiosis. In its acute form, this disease is characterized by fever, malaise, anorexia, constipation, backache, and lymphadenopathy (9). No information is available regarding the means by which the disease is transmitted. Various hypotheses concerning the source of infection, including consumption of raw fish meat, have not been substantiated.

Previous immunological studies have failed to demonstrate an antigenic relationship between *E. sennetsu* and other rickettsiae of medical importance. Recently, however, a serological relationship between *E. sennetsu* and *Ehrlichia canis*, the causative agent of canine ehrlichiosis, was demonstrated (12). Although these two organisms are pathogens of two widely separated animal species, humans and dogs, their morphological appearances and their predilection for blood monocytes are similar.

*E. sennetsu* has previously been grown in cell cultures by using HeLa cells (19), human amniotic membrane-derived FL cells (8), and African green monkey kidney cells (1). None of these systems produced sufficient quantities of the organism for immunoserological studies (N. Tachibana and E. Kusune, abstr., Second Dept. Med., Miyazaki Med. Sch., Miyazaki, Japan). Consequently, cyclophosphamide-treated mice rather than in vitro cultures have been used for the production of *E. sennetsu* antigens (20).

Primary canine blood monocyte cultures were initially adapted for the cultivation of *E. canis* (10). Subsequently, blood monocyte cultures proved to be suitable for the cultivation of *Neorickettsia helminthoeca* (2), *Rickettsia rickettsii* (3–5), and (15). The use of this system for isolation and propagation of the latter organism provided a more efficient method for diagnosis of scrub typhus than the previous method, in which laboratory mice were used; this method required up to 3 months to demonstrate the organism.

Recently, *E. sennetsu* was successfully propagated in primary human blood monocyte cell cultures in our laboratory (6). However, the availability of cultures of primary canine blood monocytes, which are routinely used by workers in our laboratory for the production of *E. canis*, prompted us to investigate the suitability of these more easily obtainable cells for propagation of *E. sennetsu*. The infectivity of this organism for dogs and its suitability for serodiagnosis of human sennetsu rickettsiosis were also studied.

**MATERIALS AND METHODS**

**Organisms.** *E. sennetsu*, which was obtained from the American Type Culture Collection, Rockville, Md., was first propagated in our laboratory in primary human blood monocyte cultures. The organisms derived from the second passage of these cultures were used to infect primary canine blood monocyte cultures.

**Canine blood monocyte cultures.** The criteria used in the selection of healthy adult dogs of various breeds to serve as donors of normal blood monocytes and serum included size (27 kg or more) and a lack of serum antibodies to *E. canis* and *E. sennetsu*, as determined by indirect fluorescent antibody (IFA) tests (6, 13). Selected donor dogs were then dewormed, vaccinated against distemper, hepatitis, leptospirosis, and parvovirus, and housed individually in tick-free isolation units. Normal canine blood monocyte cultures were established by collecting 40 ml of blood aseptically from the jugular vein of a donor dog in a sterile plastic syringe containing 2,000 USP units of sodium heparin and 20 ml of sterile 2.5% dextran (molecular weight, 500,000) in physiological saline solution. The dextran solution was used to accelerate the sedimentation of erythrocytes. After col-
lection, the needle was replaced with a sterile needle and plastic sheath. The blood was thoroughly mixed with the heparin-dextran solution by gentle agitation. The syringe was then clamped to a ring stand with the needle end up. The syringe was kept in this position for 1 h at room temperature to allow complete sedimentation of the erythrocytes. With the syringe remaining in an upright position, the needle was bent downward at a 45° angle, and 5 ml of the leukocyte-rich plasma was transferred to 25-cm² tissue culture flasks.

The cultures were incubated at 37°C for 48 h to allow the monocytes to attach to the flasks. The fluid portion of each culture was discarded, and the cells were washed twice by adding 2 ml of Hanks balanced salt solution to each flask; 5 ml of culture medium (Earle’s minimal essential medium containing Earle’s salts supplemented with 1% L-glutamine [200 mM] and 20% heat-inactivated autologous serum) was added to each flask. The culture medium was replaced every 48 to 72 h. At 7 to 10 days after the start of culture, a complete cell monolayer was achieved. At this time, the supernatant culture medium was removed, and each flask was inoculated with 2 ml of supernatant containing 10⁶ E. sennetsu-infected cells derived from human blood monocyte cultures (6). The flasks were incubated at 37°C for 30 min, and then 3 ml of fresh medium was added to each flask. The cultures were then maintained as described above.

Monocytes found free in the supernatant of each flask were examined daily for evidence of infection with E. sennetsu. To do this, 1 ml of supernatant was transferred to a vial (diameter, 14 mm) provided with a 12-mm cover slip. The vial was then centrifuged at 600 x g for 10 min. The cover slip was removed, stained by using the Wright-Giemsa method, mounted cell side down, and examined. The supernatant from cultures containing approximately 10⁶ infected monocytes was used to infect another set of normal cultures initiated 7 to 10 days earlier. The procedures described above were repeated continuously.

**Determination of cell infectivity.** To quantitate the levels of infectivity of E. sennetsu in primary canine monocyte cultures, six cultures representative of each passage were incorporated into Leighton tubes containing cover slips. Each culture monolayer was inoculated with 2 x 10³ E. sennetsu-infected monocytes. To determine the average maximum growth at each passage, representative cultures were terminated on day 14 of incubation, and the average percentages of infected monocytes were determined by microscopic examination of Wright-Giemsa-stained cover slips. To evaluate the growth pattern of the organism within a single passage, 33 Leighton tubes containing E. sennetsu-infected cell monolayers at passage 11 were established. After 24 h of incubation and every 48 h thereafter for a total of 21 days, three cultures were terminated and examined as described above.

**Electron microscopy.** Supernatant from highly infected cultures (approximately 80% infected) was removed and replaced with 2 ml of 0.02% EDTA. Flasks were agitated until all of the cells were liberated from the surfaces. The suspended cells were placed into a conical tube and centrifuged at 600 x g for 15 min. The cells were washed twice in phosphate buffer (pH 7.4), centrifuged at 600 x g for 10 min, and fixed in 2 ml of a 3% glutaraldehyde solution for 1 h at 4°C. The remaining fixation and examination methods used were those used in the study of *Ehrlichia equi* (14).

**Dog inoculation.** One beagle (dog B-2) and one German Shepherd dog (dog GS-1) were inoculated with 5-ml suspensions containing 1.2 x 10⁶ E. sennetsu-infected cells obtained from cell culture passages 3 and 11, respectively. A beagle (dog B-1) inoculated with a corresponding quantity of cells from a normal noninfected monocyte culture served as a control. Each dog received 3 ml of the inoculum intravenously and 2 ml intramuscularly. A second German Shepherd dog (dog GS-2) was inoculated with 20 ml of whole blood (10 ml intravenously and 10 ml intramuscularly) from dog B-2 90 days after inoculation of the latter. Temperature, hematological values, and clinical signs were recorded 4 days prior to inoculation and daily thereafter for 30 days. On days 14, 16, and 18 following inoculation, 20 ml of blood was drawn from each dog for initiation of monocyte cell cultures. The cultures were maintained as described above and were examined daily for the presence of E. sennetsu.

**IFA test.** The procedure used to prepare E. sennetsu antigen slides was similar to the procedure described for *E. canis* by Ristic et al. (13). When approximately 60 to 80% of the cultured canine monocytes became infected with E. sennetsu, the cells were harvested and concentrated for use as an antigen in the IFA test. Culture supernatant was removed, and cell monolayers were harvested by adding 2 ml of 0.02% sodium EDTA to each flask, followed by agitation on a clinical rotator at 170 oscillations per min for 30 min. The cell suspensions were then pooled and centrifuged at 600 x g for 15 min. After removal of the supernatant, the cells were suspended in 20 volumes of 0.15 M phosphate-buffered saline (pH 7.2), washed three times, and centrifuged at 600 x g for 15 min. The cell pellet was then suspended in 5 volumes of 2.0% bovine serum albumin in 0.15 M phosphate-buffered saline. Drops (10 µl) of the antigen suspension were placed onto acetone-cleaned microscope slides. The slides were dried at 37°C for 1 h, wrapped in lint-free tissue, and stored at -20°C.

Prior to use, the antigen slides were thawed at room temperature in a vacuum desiccator jar for 1 h and fixed in fresh acetone for 15 min at room temperature. Circles (5 mm) were drawn around individual antigen spots by using a Tech Pen (Mark-Tex Corp., Englewood, N.J.).

Human sera from patients diagnosed as having sennetsu rickettsiosis on the basis of clinical symptoms, mouse inoculations, and IFA serology were provided by N. Tachibana, Miyazaki Medical School, Miyazaki, Japan (12). A total of 200 coded serum samples were received from D. L. Huxsoll, U.S. Army Medical Research Unit, Kuala Lumpur, Malaysia. These sera originated from patients residing in various regions of Malaysia. These patients were considered to be suffering from fever of unknown origin after numerous attempts to identify the cause of illness failed. In addition, 200 coded serum samples from apparently healthy individuals not indigenous to the region were also received. All sera were examined for antibodies against E. sennetsu by using the IFA test. Sera of healthy volunteers from our laboratory served as negative controls. Sera from E. sennetsu-inoculated and uninoculated negative control dogs were also examined by the IFA test. Fluorescein-conjugated goat anti-human immunoglobulin G and rabbit anti-dog immunoglobulin G were obtained commercially.

**RESULTS**

Sequential microscopic examination of cultured canine blood monocytes stained with Wright-Giemsa and IFA techniques revealed various growth forms of *E. sennetsu*. At 7 days after infection, the organism (appearing as minute ovoid bodies) was detected in approximately 8% of the monocytes present in the culture supernatant (Fig. 1A). By day 14, approximately 18% of the cells were infected with the organism. At 10 days after infection of the first set of
subcultures, numerous organisms relatively evenly distributed throughout the cytoplasm were present in nearly 40% of the monocytes, as revealed by the IFA method (Fig. 1B). Some of the growth forms resembled classic morulae of *E. canis* in canine blood monocytes (Fig. 1C, arrows). This growth stage was followed by apparent disintegration of monocytes and gradual release of the organism into the culture medium. Most frequently, however, extracellularly occurring organisms remained interconnected by cell debris or in the form of loosely packed clusters (Fig. 1D). More prolific growth of the organism was noted in each subsequent passage, with some cell cultures reaching an infection rate of approximately 95% by passage 14. At this time, the cytoplasm of many cells was completely obliterated by the organism.

Based upon sequential microscopic examination of cultures representing passage 11, the average percentages of infected monocytes are shown in Fig. 2A. It is apparent that the most rapid growth phase of the organism occurred during days 7 through 15. A collective evaluation of the average maximum percentages of infected monocytes for 15 consecutive culture passages of *E. sennetsu* is presented in Fig. 2B. Our results indicate that the organism attained its maximal growth at approximately culture passage 7 and, with slight variation, maintained that level through subsequent passages.

Electron microscopic examination revealed that singly occurring organisms or inclusion bodies containing two or more organisms were always contained within an alveolar membrane (Fig. 3). Individual organisms were enclosed by an outer rippled limiting membrane (Fig. 3C, arrow). Some of the inclusion bodies were relatively large and resembled *E. canis* initial bodies or morulae. The sizes of individual organisms ranged between 0.4 and 1.0 μm wide and between 0.4 and 2.5 μm long.

With the exception of a slight increase in temperature (39.4 to 40.0°C) between days 11 and 16 postinfection, no clinical manifestation was observed in any of the inoculated dogs.

*E. sennetsu* was reisolated in monocyte cultures derived...
from the blood of one inoculated dog (dog B-2) on day 16 postinfection and the blood of two dogs (dogs GS-1 and GS-2) on day 18 postinfection and maintained through subpassages. Further attempts to reisolate the organism were not made. Monocyte cultures prepared from control dog B-1 showed no evidence of infection with the agent.

*E. sennetsu* antigen produced in canine blood monocyte cultures was suitable for the detection and titration of human and canine anti-*E. sennetsu* antibodies by the IFA technique. Of the five human serum samples obtained from sennetsu rickettsiosis patients, one reacted at a titer of 1:640, two reacted at a titer of 1:320, and two reacted at a titer of 1:80. Of 200 sera from patients with fever of unknown origin, 58 (29%) were positive for antibodies reactive with *E. sennetsu*. None of the 200 sera from apparently healthy individuals reacted in the test. The first reaction with sera from dogs infected with culture-derived *E. sennetsu* was noted on day 14 postinoculation at a titer of 1:10 and reached a maximal titer of 1:80 on day 35. Dog GS-2, which was infected with carrier blood from dog B-2, attained a maximal titer of 1:640 at 21 days postinfection. All control sera were negative at a serum dilution of 1:10.

**DISCUSSION**

The results of this study demonstrated that *E. sennetsu* can be readily propagated in canine monocyte cultures derived from the peripheral blood of normal dogs. Assuming that the host cells were nonactivated monocytes, it appears that canine monocytes possess the cell surface receptors that are required for the attachment and penetration of *E. sennetsu* (17). It has been hypothesized that once organisms such as *E. sennetsu*, *E. canis*, and chlamydiae are inside a cell, they evade the degradation process due to lysosomal enzymes by segregation within a cell vacuole (7).

Although various types of media were not compared in this study, minimal essential medium supplemented with 1% L-glutamine (200 mM) and 20% autologous serum provided all of the metabolic requirements for maintenance of the host cells and for the continued growth and replication of *E. sennetsu*. The use of autologous serum was necessary to achieve confluent cell monolayers. Confluency was not always attained when homologous or heterologous sera (e.g., fetal bovine serum) were used.

Light microscopy of *E. sennetsu* revealed growth forms similar to those of *E. canis* (18). Electron microscopy showed that individual organisms or clusters of organisms were contained within a vacuole membrane, resembling the initial bodies (immature inclusions) and morulae (mature inclusions) of *E. canis*. However, the outer rippled membrane of *E. sennetsu* more closely resembles that of *E. equi* than that of *E. canis* (14). The *E. sennetsu* organisms exhibited a considerable degree of pleomorphism, with the appearance of forms similar to those observed by Anderson et al. (1). Sequential observation revealed that *E. sennetsu* multiplied by binary fission and ultimately filled the cytoplasm of the cells, leading to host cell rupture. The freed organisms remained attached to remnants of the cell cytoplasm and, for an undetermined length of time, maintained the capability to invade new host cells and repeat the multiplication process. A similar phenomenon has been described for *Rickettsia tsutsugamushi* (21). As the latter rickettsiae are released from their host cells, they remain firmly attached to host cell components which form a membrane around individual organisms. Evidence suggests that the host membrane is involved in protection of the rickettsial cell wall since, when the rickettsiae are removed by physical or chemical methods, they are usually rendered inactive (16). A possible role of the host cell material for *E. sennetsu* was not determined in this study.

Determinations of the percentages of infectivity during consecutive culture passages of *E. sennetsu* indicated that maximal growth was attained at approximately passage 7. The pattern of growth of the organism within a single passage revealed a progressive increase in infected monocytes until approximately day 14 of incubation. Thereafter, the level of infected monocytes remained relatively stable.

Morphological and antigenic resemblance between *E. canis* and *E. sennetsu* prompted the recent reclassification of the latter agent from species incertae sedis to *E. sennetsu* in the genus *Ehrlichia* in Bergy's *Manual of Systematic Bacteriology* (11). The members of this genus seem to represent a unique group of agents which differ from other groups of rickettsiae.

The propagation of *E. sennetsu* in canine monocytes provides an abundant supply of antigen for use in the IFA test and various other studies. Although the number of sera from human patients infected with *E. sennetsu* has been limited, the IFA test with canine monocyte-derived antigen appears to be a useful and specific procedure for the diagnosis of sennetsu rickettsiosis in humans (12). By using this test system, workers in our laboratory detected anti-*E. sennetsu* antibodies in 29% of 200 serum samples obtained from patients with fever of unknown origin. These data and the recent isolation of an agent resembling *E. sennetsu* from the blood of three seropositive patients suggest that infec-
tions with \textit{E. sennetsu} or related agents or both may exist in regions of Southeast Asia other than Japan (A. I. Cole, personal communication). The nature of these recently isolated agents is currently being investigated.

Dogs inoculated with the cell-cultured organism showed transient moderate pyrexia. No other clinical evidence of disease was observed. Primary inoculation with culture-derived \textit{E. sennetsu} induced a maximum IFA titer of 1:80, whereas subinoculation by using whole blood led to a maximum titer of 1:640. The slightly higher titer following blood inoculation from an infected donor dog will be the subject of a future investigation. Although we recognize that the number of dogs used in this study was small, it is nevertheless worth noting that the dogs never succumbed to challenge and that organisms were subsequently cultured from their blood. Thus, it appears that dogs could serve as a good model for epidemiological research on human sennetsu rickettsiosis. If additional evidence can be generated regarding the persistence of latent \textit{E. sennetsu} infections in inoculated dogs, such evidence would suggest that these animals may be inapparent hosts for the perpetuation of this agent. This hypothesis could be substantiated by obtaining sera from dogs in Western Japan, where the disease is most prevalent, and examining these sera for the presence of antibodies to \textit{E. sennetsu} by using the IFA technique. The possible existence of inapparent carriers could provide much needed information regarding the epidemiology of sennetsu rickettsiosis and aid in determining the means by which the organism is transmitted. A relationship has been suggested between the eating of certain types of seasonal fish and sennetsu rickettsiosis. However, Tachibana and Kusune (20) observed that only persons who actually caught the fish developed the disease. This evidence suggests a possible role for a vector (e.g., ticks) in the cycle of the development and transmission of \textit{E. sennetsu}.

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LITERATURE CITED


