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Reisolation of *Ehrlichia canis* from Blood and Tissues of Dogs after Doxycycline Treatment

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We present evidence that supports the carrier status of dogs experimentally infected with *Ehrlichia canis* after treatment with doxycycline. Canine ehrlichiosis was induced in five dogs by intravenous inoculation with *E. canis*-infected DH82 cells. All animals developed mild clinical signs of transient fever, body weight loss, thrombocytopenia, and increased gamma globulin levels in plasma. An indirect fluorescent-antibody test (IFA) revealed that all dogs had seroconverted (titer, 5,120) by day 10 postinoculation (p.i.). *E. canis* was reisolated from blood samples collected at intervals throughout the 2-month period p.i. Doxycycline was administered orally once daily at 10 mg/kg of body weight per day for 1 week starting at 2 months p.i. Following treatment, gamma globulin levels in plasma were decreased. At necropsy on days 54 to 59 after the start of treatment, spleen, liver, kidney, and lymph nodes were collected for *E. canis* culture and histopathologic examination. Although the dogs did not show significant clinical signs during or after treatment with the antibiotic, *E. canis* was reisolated from the blood and tissue samples of three of five dogs. A 16-fold reduction in IFA titer was noted in two dogs which were negative for *E. canis* reisolation at day 49 after the start of treatment, whereas a zero- to fourfold reduction in IFA titer was seen in the remaining three dogs. Western immunoblot reactions to higher-molecular-size *E. canis* antigens in the sera of two dogs which were negative for *E. canis* on blood culture decreased, whereas they remained continuously high or only transiently decreased for the duration of the study for antigens in the sera of three dogs from which *E. canis* was reisolated. Histopathologically, prominent plasmacytosis in the kidney cortex was present in three dogs from which *E. canis* was reisolated, whereas the kidney cortices of two dogs had moderate to minor plasmacytosis. These findings pose questions regarding the efficacy, dosage, and duration of doxycycline treatment in dogs with *E. canis* infection. In addition, it was shown that IFA and Western immunoblotting may aid in assessing the efficacy of antibiotic therapy when definitive reisolation procedures are not readily available.

Canine ehrlichiosis, which is caused by *Ehrlichia canis*, was originally described in Algeria in 1935 (10), and it has now been reported throughout most of the world, but particularly in tropical and subtropical regions (18, 19, 33). The disease is characterized by fever, depression, anorexia, and body weight loss in the acute phase, with laboratory findings of thrombocytopenia and gammaglobulinemia (5, 13). Hemorrhages, epistaxis, edema, and hypotensive shock, which lead to death in the chronic phase, are often exacerbated by superinfection with other organisms (5, 13, 20). A subclinical phase is often seen (8, 17, 27).

A wide variety of chemotherapeutic agents have been suggested for use in the treatment of canine ehrlichiosis. Various sulfonamides (3, 4, 7, 21) and antibiotics including penicillin (12), chloramphenicol (4), tetracyclines (2, 3, 5, 11, 22, 34), and imidocarb dipropionate (1, 23, 29) have been used in treatment regimens with varying degrees of success. Tetracycline or its derivatives are believed to be the most effective clinically and are the most widely used antibiotics for the treatment of canine ehrlichiosis (2, 3, 5, 11, 22, 34).

Doxycycline is a synthetic derivative of methacycline and is also known as α-6-deoxy-5-oxytetracycline (15). The mode of action of doxycycline is similar to those of the other tetracyclines, i.e., the inhibition of bacterial protein synthesis. Doxycycline differs from the tetracyclines in that it has a high degree of lipid solubility and is more completely absorbed from the gastrointestinal tract than oxytetracycline. After absorption, the antibiotic is 30% protein bound and penetrates well into tissues. The renal clearance of doxycycline is less than that of oxytetracycline, which may be attributed to its high degree of lipid solubility. Because of the high degree of absorption and slow excretion rates, doxycycline has a long half-life in serum, approximately 19.5 h, in comparison with a 9.5-h half-life for oxytetracycline (15). The current recommendation for treating canine ehrlichiosis is the administration of doxycycline at 5 to 10 mg/kg of body weight orally (or intravenously) every 12 to 24 h for 7 to 10 days (13).

Investigations on the efficacies of doxycycline (29–31) and other derivatives of tetracycline (2, 3, 5, 11, 22, 34) against *E. canis* infections have been carried out in the past. Because of the nonavailability of appropriate techniques or because of the laborious nature of the available techniques, it has not been confirmed whether tetracyclines completely eliminate *E. canis* infection. In the present study, the efficacy of doxycycline in the treatment of experimental canine ehrlichiosis was assessed by cell culture reisolation, indirect fluorescent-antibody test (IFA), Western immunoblot analysis (WI), plasma gamma globulin concentration determination, and histopathologic examination of tissue specimens obtained at necropsy.

MATERIALS AND METHODS

Culturing of *E. canis*. The *E. canis* Oklahoma isolate was cultured in a dog macrophage cell line (DH82) in minimum essential medium (MEM; GIBCO, Grand Island, N.Y.) containing 10% fetal bovine serum (FBS; Atlanta Biologicals,

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Norcross, Ga.) and 2 mM L-glutamine (GIBCO) in 5% CO_2–95% air as described previously (26).

Experimental infection and treatment of dogs. Five 1- to 2-year-old German shepherd-mixed breed dogs were purchased from Biomedical Associates, Inc., Friedensburg, Pa. The dogs were either colony bred or originally acquired from a pound by Biomedical Associates after a veterinarian's health certification and rabies vaccination. They had been quarantined in the facility for more than 1 year. During the quarantine period, each dog was vaccinated against distemper, hepatitis and parvovirus infections, and leptospirosis, and several dewormings were done. Dogs weighed 24 to 36 kg at the beginning of the study. At 15 days before and on day 0 of the experiment, all dogs were seronegative for *E. canis*, as determined by IFA. Each dog was inoculated intravenously with 10^7 *E. canis*-infected DH82 cells suspended in 5 ml of MEM. Rectal temperature, appetite, attitude, and other abnormalities were monitored daily. At 2 months postinoculation (p.i.), doxycycline hyclate (Mutual Pharmaceutical Co., Inc., Philadelphia, Pa.) was administered orally to the dog at 10 mg/kg daily for 1 week. Heparinized blood samples (30 ml) were collected from the jugular or cephalic vein on days 0, 7, 21, 28, and 35 p.i. and on days 0 (63 days p.i.), 7, 21, 49, and 54 after the start of the treatment (posttreatment [PT]). Complete blood counts determined with a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.), measurement of the plasma gamma globulin concentration by the radial immunodiffusion method (VMRD, Inc., Pullman, Wash.), IFA, WI, and reisolation of *E. canis* in cell culture were carried out on the blood samples.

IFA. IFA was performed as described previously (26). Briefly, *E. canis*-infected cells suspended in MEM were air dried and acetone fixed in 12-well Teflon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.)-coated multiwell slides (Cel-Line Associates, Newfield, N.J.). Serial twofold dilutions of the test plasma sample in phosphate-buffered saline (PBS; 10 μl), starting at a 1:20 dilution, were placed in the wells of antigen-coated IFA slides. The slides were incubated in a humidified incubator at 37°C for 30 min. After three successive rinses in PBS containing 0.002% Tween 20, 10 μl of fluorescein-conjugated goat anti-dog immunoglobulin G (United States Biochemical Corporation, Cleveland, Ohio) diluted 1:200 in PBS was added to each well. The slides were incubated, washed, blotted, and examined with an epifluorescence microscope.

Purification of *E. canis*. *E. canis* was purified from five to eight flasks (150 cm^2) of infected DH82 cells as described previously (26). Briefly, the cultured cell suspensions were centrifuged to a pellet, resuspended in MEM, and disrupted by a repeated and controlled weak sonication. Unbroken cells and nuclei were sedimented by centrifugation, and the supernatant was centrifuged at 15,000 × *g* for 10 min at 4°C. The pellets were resuspended in 1.5 ml of 2× PBS (2 mM KH_2PO_4, 6 mM Na_2HPO_4, 2 mM KCl, 136 mM NaCl [pH 7.4]). The suspension was applied to the top of a packed chromatography column (20 by 2 cm) of Sephacryl S-1000 (Pharmacia, Uppsala, Sweden) and was eluted with 2× PBS. The flowthrough fractions collected contained ehrlichial organisms.

WI. Purified *E. canis* antigens (40 μg per lane) were solubilized in sodium dodecyl sulfate (SDS)–β-mercaptoethanol sample buffer and were separated by SDS–12.5% polyacrylamide slab gel electrophoresis. WI was performed on dog sera collected before and after treatment (diluted 1:100) and on a 1:1,000 dilution of alkaline phosphatase-conjugated, affinity-purified anti-dog immunoglobulin G (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) as described previously (24).

Isolation and culture of *E. canis*. Heparinized blood samples (30 ml) from each dog were centrifuged at 2,500 × *g* for 5 min. Plasma was collected and was stored at −20°C until use. The buffy coat at the interface of the plasma and erythrocytes was harvested and overlaid on Histopaque 1077 (Sigma Chemical Co., St. Louis, Mo.), and the combination was centrifuged at 2,000 × *g* for 15 min. The interface containing the mononuclear cell fraction was harvested and washed in MEM with 1% of antibiotic-antimycotic mixture (10,000 U of penicillin G per ml, 10,000 μg of streptomycin sulfate per ml, and 25 μg of amphotericin B per ml [GIBCO]). The pellet was resuspended in 3 ml of MEM and the combination was mixed to a homogeneous suspension. A 1-ml aliquot of the mononuclear cell fraction was overlaid on a semiconfluent monolayer of canine macrophage DH82 cells (32) in MEM containing 10% heat-inactivated FBS and 2 mM L-glutamine, and the mixture was cultured in 5% CO_2–95% air at 37°C.

The liver, spleen, kidney, and lymph node samples that were aseptically removed at necropsy were immediately soaked in MEM containing the 1% antibiotic-antimycotic mixture. The tissues were smashed with the blunt end of a pestle through a cell collector (Cellector; Thomas Scientific, Swedesboro, N.J.) to release the cells. The cell suspension derived from approximately 3 g of tissue was overlaid on Histopaque 1077, and the interface was collected, washed, overlaid on a monolayer of DH82 cells, and cultured as described above. At 2-day intervals, inoculated DH82 cells were dissociated from the bottom of the flask by tapping the flask, and an aliquot (0.2 ml) was centrifuged onto a glass slide in a cytocentrifuge (Cytospin 2; Shandon, Inc., Pittsburgh, Pa.). The cells were stained with Diff-Quik stain (Baxter Scientific Products, Obetz, Ohio) and were examined for the presence of *E. canis* by light microscopy. Cultures were considered negative if *E. canis* was not reisolated by up to 2 months postculture.

Postmortem examination. At days 54 to 59 PT, a necropsy was performed immediately following euthanasia of the animals by intravenous injection of Uthol (5 g of pentobarbital sodium, 40% isopropyl alcohol, 2% propylene glycol, 0.03% edetate sodium, 2% benzyl alcohol; 1 ml/5 kg of body weight; The Butler Company, Columbus, Ohio). All of the organs were examined grossly for abnormalities. Samples of spleen, liver, kidney, and lymph nodes were collected aseptically for *E. canis* culture. For histopathologic examination, specimens from these organs were fixed in 10% neutral buffered formalin, processed for paraffin embedding, and stained with hematoxylin and eosin. Histopathology slides were read by an observer blinded to their source.

RESULTS

Clinical signs. The rectal temperatures and body weights of all dogs were very similar at 1 week preexposure and at day 0 postexposure. All dogs developed mild clinical signs of transient fever, weight loss, thrombocytopenia, and increased plasma gamma globulin concentrations within 2 to 4 weeks p.i. After treatment, in comparison with the start of treatment (day 0 PT), reductions in the rectal temperatures of and gamma globulin concentrations in all dogs were observed (Fig. 1). A loss of body weight occurred in only one dog (dog 320). A stable increase in thrombocyte counts was seen in all except one dog (dog 340) (Table 1). Gamma globulin concentrations again increased by days 54 to 57 PT in three dogs (dogs 303, 307, and 340) (Fig. 1).

IFA. IFA revealed that all dogs were negative for *E. canis* at

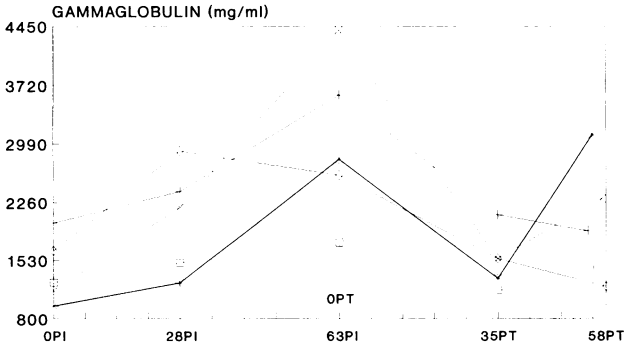

FIG. 1. Plasma gamma globulin concentrations of dogs infected with *E. canis* before and after doxycycline treatment. +, dog 011; *, dog 340; □, dog 307; ×, dog 303; ◇, dog 320. PI, postinoculation; PT, posttreatment.

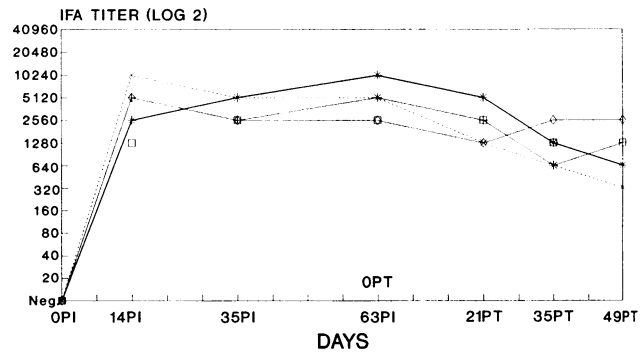

FIG. 2. Immunoglobulin G antibody titers (reciprocal dilutions) against *E. canis* determined by IFA before and after doxycycline treatment. +, dog 011; *, dog 303; □, dog 307; ×, dog 320; ◇, dog 340. PI, postinoculation; PT, posttreatment.

days −7 and 0 p.i. but became seropositive between days 2 and 6 p.i. The titers reached a plateau at day 10 p.i. and remained high on day 0 PT (day 63 p.i.). A two- to fourfold decrease in the antibody titer in all dogs was observed by days 21 to 35 PT. A 16-fold decrease in titer was noted at day 49 PT in two dogs (dogs 303 and 320), whereas a zero- to fourfold decrease in titer was observed in the remaining three dogs. In two dogs (dogs 340 and 011), the titer was again increased at days 35 and 49 PT and reached titers of 1:5,120 and 1:2,560, respectively, at day 49 PT (Fig. 2).

WI. The major proteins of purified *E. canis* identified by SDS-polyacrylamide gel electrophoresis and the absence of a WI reaction of the sera from dogs inoculated with either *E. canis*-infected DH82 cells or uninfected DH82 cells to DH82 cell antigens were shown previously (25). The results of WI of the development of antibodies against *E. canis* antigens before and after treatment are given in Fig. 3. A negligible reaction of antibodies and antigens was seen for all dogs on day 0 p.i., whereas all dogs developed antibodies which reacted with both low-molecular-mass polypeptides (21, 30, 31, and 40 KDa) and higher-molecular-mass polypeptides (47, 64, and 78 KDa) of *E. canis* by day 14 p.i. The intensities of the reactions and the numbers of reacting polypeptide antigens were significantly increased for all dogs by day 63 p.i. (day 0 PT). The reactions of antibodies with antigens of *E. canis* were weakened for some higher-molecular-mass polypeptides (47, 64, and 78 KDa) for dogs 011, 303, and 340 by 3 to 5 weeks PT. This weakened reactivity persisted for 2 to 3 weeks, but after that a good reaction was again observed for serum from one dog (dog 340). The serum of dog 307 maintained similar patterns of reactivity throughout the post-antibiotic treatment period. In the sera of two dogs (dogs 303 and 320), reactivity became significantly weaker for higher-molecular-mass polypeptides (>40 KDa) at day 49 PT.

Cell culture reisolation of *E. canis* from peripheral blood mononuclear cell fractions and tissues. *E. canis* was consistently isolated from the mononuclear cell fraction of all dogs before treatment (except day 0 p.i.), whereas after treatment, the organisms were isolated from blood (Fig. 4) as well as several tissues of three dogs (dogs 011, 307, and 340) (Tables 2 and 3). It was noted that in these three dogs, the time taken for the organisms to show up in culture was longer (34 to 51 days) for isolates obtained on day 7 PT than for those obtained before treatment (13 to 34 days). Once samples became

TABLE 1. Period of time that platelet counts were less than 100,000/μl in the study dogs

Dog no.	Before treatment		After treatment		
	Platelet count (10^3/μl) at day 0 p.i.	No. of wks platelets were <100,000 per μl/total no. of wks	Platelet count (10^3/μl) at days 0 and 7 PT		No. of wks platelets were <100,000 per μl/total no. of wks
			Day 0	Day 7	
011	130	2/9	162	200	0/8
303	211	8/9	60	212	0/8
307	186	1/9	198	245	0/8
320	105	3/9	127	140	0/8
340	357	0/9	131	211	2/8

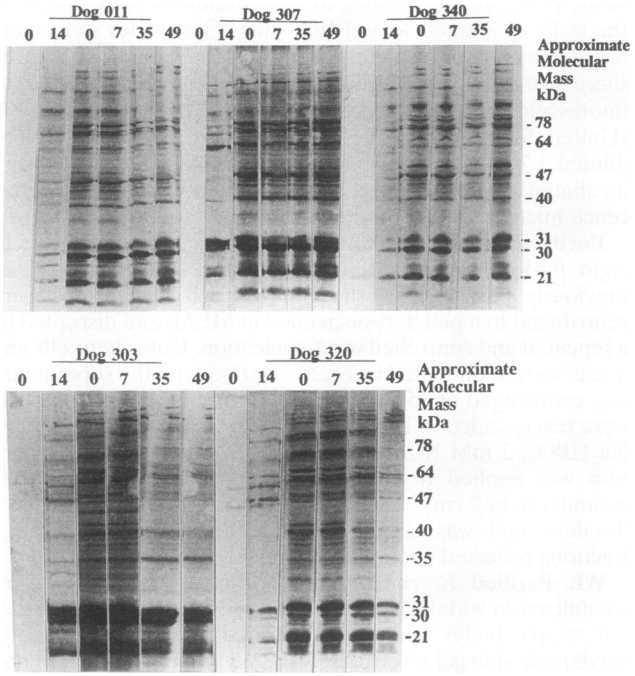

FIG. 3. WI of dog sera for *E. canis* antigen separated by SDS–12.5% polyacrylamide gel electrophoresis. Sera were collected at 0 and 14 days p.i. and 0, 7, 35, and 49 days PT.

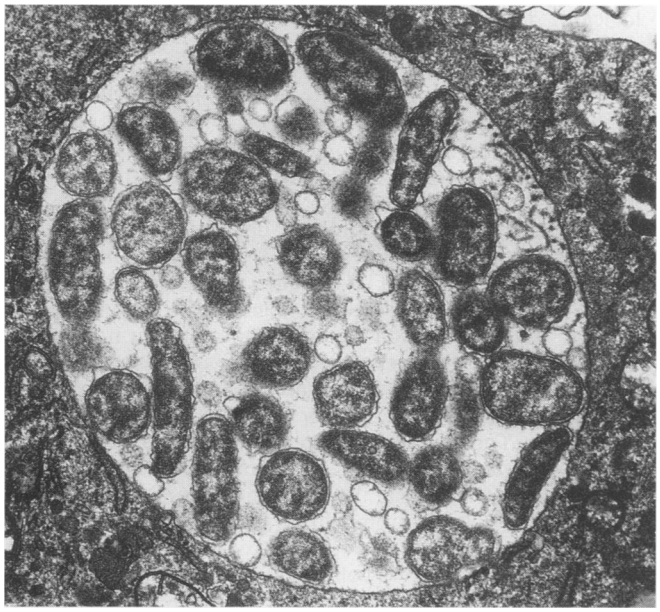

FIG. 4. *E. canis* in the cytoplasm of DH82 cell culture reisolated from blood after doxycycline treatment. Magnification, ×14,000.

negative at day 7 PT, the sera of two dogs (dogs 303 and 320) remained negative.

Postmortem findings. At necropsy, there were no gross abnormalities with the exception of dog 340, which had enlarged mesenteric, cervical, and popliteal lymph nodes. The liver and spleen of this dog were enlarged and congested, and hemorrhages were present on a portion of the surfaces of these organs (Fig. 5). Histopathologically, prominent plasmacytosis was revealed in the kidney cortices of dogs 011, 307, and 340 (Fig. 6), and minor plasmacytosis was noted in dogs 303 and 320. Large secondary follicles with prominent plasma cells at the peripheries of the follicles and medullary cords were seen in the lymph nodes of all dogs; these were especially prominent in dogs 011, 307, and 340. Plasma cells were also dominant in the mantle layer around the germinal centers of the spleens of these three dogs.

DISCUSSION

Although clinical trials for evaluating the efficacy of doxycycline have been conducted in the past (29–31), this is the first study which clearly demonstrated the ineffectiveness of this antibiotic in completely eliminating *E. canis* infection. This statement is based on the application in the present study of the most sensitive new procedures for determining infection before and after treatment of the animals. Previous investigations were limited to experimental infections by using whole blood as the inoculum, natural infection, IFA for the detection of antibodies against *E. canis*, hematologic evaluations, clinical observations, blood smear examinations, or the use of short-term (24- to 72-h) cell culture isolation techniques (23, 29–31). Since *E. canis* multiplies so slowly and too few cells are present in the blood of infected animals, examination of blood smears or short-term blood cell culture may provide false-negative results. In contrast, our study was conducted under controlled experimental conditions by using a known number of cultured *E. canis* organisms, IFA, WI, hematology, clinical observations, histopathology, and the cell culture isolation technique with the continuous canine macrophage cell line DH82 to maintain cultures for up to 2 months before they are reported to be negative. In addition, culture of mononuclear cells from tissue samples of dogs following doxycycline treatment was done to test for the possible sequestration of *E. canis* in the reticuloendothelial system after clearance of the organism from the blood.

The dogs in the present study were considered to be in the subclinical phase of canine ehrlichiosis at the time of antibiotic treatment. The subclinical phase is characterized by hypergammaglobulinemia and mild thrombocytopenia with the persistent presence of *E. canis* in the blood as described previously (8, 17, 27). Tissue culture isolation of *E. canis* confirmed that all of these dogs were infected at the time of the initiation of

TABLE 2. Period of time that *E. canis* was reisolated from cultures inoculated with peripheral blood monocytes

Dog no.	Days required to cultivate *E. canis* from peripheral blood monocytes at the following sampling times:					
	0 p.i.	7 p.i.	14 p.i.	0 PT	7 PT	49 PT
011	Neg[a]	30	30	13	51	30
303	Neg	17	14	32	Neg	Neg
307	Neg	16	17	22	34	42
320	Neg	15	15	25	Neg	Neg
340	Neg	18	18	18	39	17

[a] Neg, negative at day 60 postculture.

TABLE 3. Period of time that *E. canis* was reisolated from cultures inoculated with monocytes from tissues

Dog no.	Days required to cultivate *E. canis* from tissue monocytes				
	Liver	Spleen	Kidney	Mesenteric lymph node	Popliteal lymph node
011	22	19	23	9	11
303	Neg[a]	Neg	Neg	ND	ND
307	14	12	ND	12	ND
320	ND[b]	Neg	Neg	Neg	ND
340	16	13	16	ND	15

[a] Neg, negative.
[b] ND, not done.

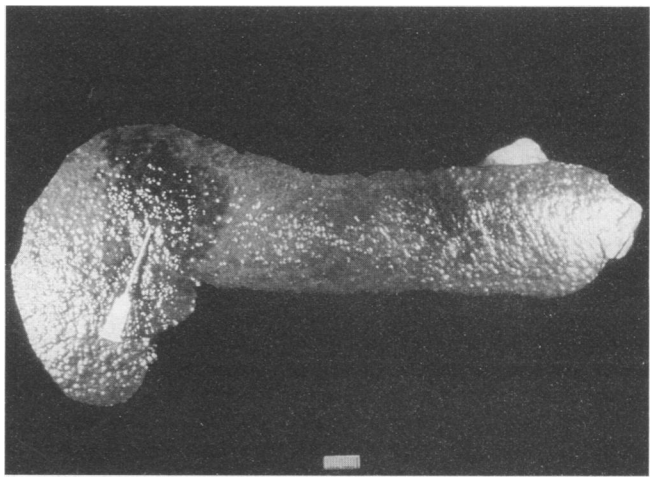

FIG. 5. Hemorrhage at 55 days PT in the spleen of dog 340 infected with *E. canis*. Bar, 1 cm.

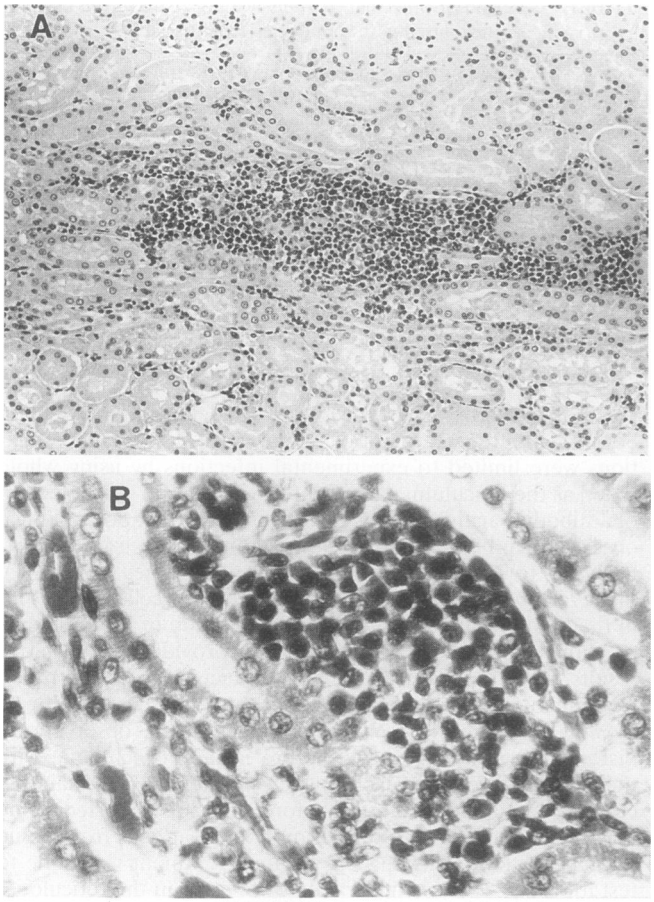

FIG. 6. Section of a kidney showing prominent plasmacytosis at 55 days PT in dog 340 infected with *E. canis*. Magnifications, ×90 (A) and ×900 (B).

treatment. Without treatment, the lack of body weight gain, a persistent high IFA titer, strong WI reactivity, low (fluctuating) platelet count, and high gamma globulin concentration are seen (5, 6, 26). There was clinical improvement in all dogs at 1 to 5 weeks after doxycycline treatment, as indicated by an increase in body weight (in one dog) and platelet count (in all dogs) and a reduction in the IFA titer (in all dogs), gamma globulin concentration (in all dogs), and antibody reactions by WI (in three dogs). Although all of the dogs remained positive for *E. canis* by IFA until euthanasia, 16-fold reductions in the titers were observed at day 49 PT in two dogs from which *E. canis* was cleared, whereas only zero- to fourfold reductions in the titers were noted in the remaining three dogs from which *E. canis* was not cleared. Platelet counts, gamma globulin concentrations, IFA, WI, and tissue plasmacytosis suggested persistent infection and the increased proliferation of *E. canis* in dog 340 toward the end of the study. The results suggest that IFA and WI done at 7 to 8 weeks PT can be used as parameters to assess antibiotic efficacy.

In our study, persistent infection was later confirmed by reisolation of *E. canis* from the blood as well as the tissues of three dogs. Histopathology of the organs examined revealed marked plasmacytosis in these three dogs. It is suggested that plasmacytosis in the tissues of dogs with persistent *E. canis* in the host may lead to a hypersensitivity reaction or to an immunologically mediated tissue destruction (5, 6, 16). Histologically, dogs with naturally occurring or experimentally induced ehrlichiosis usually have a perivascular plasma cell infiltrate in the kidneys and various other tissues, indicating a strong humoral immune response to *E. canis* (14, 28). Despite a persistently positive IFA result, WI result, and plasmacytosis in tissues until euthanasia, *E. canis* was not isolated either from the blood or from the tissues of dog 303 or 320 after day 7 PT. It is most likely that *E. canis* was cleared from these dogs or that there were too few circulating ehrlichial organisms to be detected by our detection criteria. Thus, it appears to take a long time (>8 weeks PT) for humoral immune reactions to subside, if they ever cease, and a negative serology should not be expected as a criterion of successful treatment in a reasonable time period PT. In no case was *E. canis* reisolated from tissue, while blood cultures were negative, suggesting the absence of *E. canis* sequestration in tissue after clearance from the blood. Thus, blood culture alone would seem to be sufficient for determining the persistence of *E. canis*.

Observations regarding the number of days required for the reisolation of *E. canis* from culture also indicate the transient nature of the response of dogs to doxycycline treatment. The mononuclear cell fraction collected from three dogs (dogs 011, 307, and 340) on day 7 PT took a relatively longer time to become positive compared with those collected on days 0 and 49 PT. On the basis of our laboratory observations, the time required for a culture to become positive is directly related to the number of *E. canis* organisms and/or the number of infected monocytes in the blood.

Clinical improvement of *E. canis*-infected dogs following tetracycline treatment has been reported by many investigators (2–5, 11, 22, 34), but most of them speculated about or were reluctant to question its efficacy in completely eliminating the *E. canis* organisms (2, 4, 5, 23). The results of the present study demonstrated that, under the conditions of the study, doxycycline failed to eliminate *E. canis* from three of five dogs. Considering these findings, it would be worthwhile either to conduct further experimental trials with doxycycline by using other dose-time regimens or to conduct tests with other antibiotics. Meanwhile, we recommend that dogs should be treated orally with 10 mg of doxycycline per kg of body weight twice per day for 2 to 3 weeks and be retested by IFA at 2 months posttreatment. If the IFA titer does not drop significantly (>16-fold), additional treatments will be required.

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REFERENCES

1. **Adeyanju, B. J., and O. Aliu.** 1982. Chemotherapy of canine ehrlichiosis and babesiosis with imidocarb dipropionate. J. Am. Anim. Hosp. Assoc. **18**:827–830.
2. **Amyx, H. L., D. L. Huxsoll, D. C. Zeiler, and P. K. Hildebrandt.** 1971. Therapeutic and prophylactic value of tetracycline in dogs infected with the agent of tropical canine pancytopenia. J. Am. Vet. Med. Assoc. **159**:1428–1432.
3. **Bool, P. H., and P. Sutmoller.** 1957. *Ehrlichia canis* infections in dogs on Aruba (Netherlands Antilles). J. Am. Vet. Med. Assoc. **130**:418–420.
4. **Buckner, R. G., and S. A. Ewing.** 1967. Experimental treatment of canine ehrlichiosis and haemobartonellosis. J. Am. Vet. Med. Assoc. **150**:1524–1530.
5. **Buhles, W. C., D. L. Huxsoll, and M. Ristic.** 1974. Tropical canine

pancytopenia: clinical, haematologic, and serologic response of dogs to *Ehrlichia canis* infection, tetracycline therapy, and challenge inoculation. J. Infect. Dis. **130**:357–367.
6. **Burghen, G. A., W. R. Biesel, J. S. Walker, R. M. Nims, D. L. Huxsoll, and P. K. Hildebrandt.** 1971. Development of hypergammaglobulinaemia in tropical canine pancytopenia. Am. J. Vet. Res. **32**:749–756.
7. **Carmichael, J., and R. N. T. Fiennes.** 1942. *Rickettsia* infection of dogs. Vet. Rec. **54**:3–4.
8. **Codner, E. C., and L. L. Farris-Smith.** 1986. Characterization of the subclinical phase of ehrlichiosis in dogs. J. Am. Vet. Med. Assoc. **189**:47–50.
9. **Dawson, J. E., Y. Rikihisa, S. A. Ewing, and D. B. Fishbein.** 1991. Serologic diagnosis of human ehrlichiosis using two *Ehrlichia canis* isolates. J. Infect. Dis. **163**:564–567.
10. **Donatien, A., and F. Lestoquard.** 1935. Existence in Algerie dume Rickettsia du chien. Bull. Soc. Pathol. Exot. **28**:418–419.
11. **Ewing, S. A.** 1969. Canine ehrlichiosis. Adv. Vet. Sci. Comp. Med. **13**:331–354.
12. **Farrel, R. K.** 1966. Canine rickettsiosis, p. 285–288. *In* R. W. Kirk (ed.), Current veterinary therapy, 1966–1967. Small animal practice. The W. B. Saunders Co., Philadelphia.
13. **Greene, C. E., and J. W. Harvey.** 1990. Canine ehrlichiosis, p. 404–414. *In* C. E. Greene (ed.), Clinical microbiology and infectious diseases of the dog and cat. The W. B. Saunders Co., Philadelphia.
14. **Hildebrandt, P. K., D. L. Huxsoll, and J. S. Walker.** 1973. Pathology of canine ehrlichiosis (tropical canine pancytopenia). Am. J. Vet. Res. **34**:1309–1320.
15. **Huber, W. G.** 1977. Tetracyclines, p. 929–939. *In* L. M. Jones (ed.), Veterinary pharmacology and therapeutics. The Iowa State University Press, Ames.
16. **Huxsoll, D. L., H. L. Amyx, I. E. Hemelt, P. K. Hildebrandt, R. M. Nims, and W. S. Gochenour, Jr.** 1972. Laboratory studies of tropical canine pancytopenia. Exp. Parasitol. **31**:53–59.
17. **Huxsoll, D. L., P. K. Hilldebrandt, R. M. Nims, and J. S. Walker.** 1970. Tropical canine pancytopenia. J. Am. Vet. Med. Assoc. **157**:1627–1632.
18. **Immelman, A., and C. Button.** 1973. *Ehrlichia canis* infection (tropical canine pancytopelia or canine rickettsiosis). J. S. Afr. Vet. Assoc. **44**:241–245.
19. **Klopfer, U., and T. A. Nobel.** 1972. Canine ehrlichiosis (tropical canine pancytopenia) in Israel. Refuh Vet. **29**:24–29.
20. **Kuehn, N. F., and S. D. Gaunt.** 1985. Clinical and hematologic findings in canine ehrlichiosis. J. Am. Vet. Med. Assoc. **186**:355–358.
21. **Malherbe, W. D.** 1948. The diagnoses and treatment of rickettsiosis in dogs. J. S. Afr. Vet. Assoc. **19**:135–138.
22. **Pierce, K. R.** 1971. *Ehrlichia canis*: a cause of pancytopenia in dogs in Texas. Southwest. Vet. **105**:50–51.
23. **Price, J. E.** 1980. A comparison of the efficacy of imidocarb dipropionate and tetracycline hydrochloride in the treatment of canine ehrlichiosis. Vet. Rec. **107**:275–277.
24. **Rikihisa, Y.** 1991. Cross-reacting antigens between *Neorickettsia helminthoeca* and *Ehrlichia* species, shown by immunofluorescence and Western immunoblotting. J. Clin. Microbiol. **29**:2024–2029.
25. **Rikihisa, Y., S. A. Ewing, and J. C. Fox.** Western immunoblot analysis of *Ehrlichia chaffeensis*, *E. canis*, and *E. ewingii* infection of dogs and humans. J. Clin. Microbiol, in press.
26. **Rikihisa, Y., S. A. Ewing, J. C. Fox, A. G. Siregar, F. H. Pasaribu, and M. B. Malole.** 1992. Analysis of *Ehrlichia canis* and a canine granulocyte *Ehrlichia* infection. J. Clin. Microbiol. **30**:143–148.
27. **Stephenson, E. H., E. R. Clothiew, and M. Ristic.** 1975. *Ehrlichia canis* infection in a dog in Virginia. J. Am. Vet. Med. Assoc. **172**:63–64.
28. **Troy, G. C., J. C. Vulgamott, and G. H. Turnwald.** 1980. Canine ehrlichiosis: a retrospective study of 30 naturally occurring cases. J. Am. Anim. Hosp. Assoc. **16**:181–187.
29. **Van Heerden, J.** 1982. A retrospective study on 120 natural cases of canine ehrlichiosis. J. S. Afr. Vet. Assoc. **53**:17–22.
30. **Van Heerden, J., and A. Immelman.** 1979. The use of doxycycline in canine ehrlichiosis. J. S. Afr. Vet. Assoc. **50**:241–244.
31. **Van Heerden, J., F. Reyers, and C. G. Stewart.** 1983. Treatment and thrombocyte levels in experimentally induced canine ehrlichiosis and canine babesiosis. Onderstepoort J. Vet. Res. **50**:267–270.
32. **Wellman, M. L., S. Krakowka, R. M. Jacobs, and G. J. Kociba.** 1988. A macrophage-monocyte cell line from a dog with malignant histiocytosis. In vitro cell. Dev. Biol. **24**:223–229.
33. **Wilkins, J. H., R. S. T. Bowden, and G. T. Wilkinson.** 1967. A new canine syndrome. Vet. Rec. **81**:57–58.
34. **Willder, A. G.** 1977. Prophylactic use of tetracycline for tropical canine pancytopenia. Vet. Rec. **101**:15.