JOURNAL OF CLINICAL MICROBIOLOGY, June 1996, p. 1429–1432 Vol. 34, No. 6
0095-1137/96/$04.00+0

Isolation of *Ehrlichia canis* from Dogs following Subcutaneous Inoculation

STEPHEN D. GAUNT,[1]* RICHARD E. CORSTVET,[2] CINDY M. BERRY,[1] AND BOB BRENNAN[3]

Departments of Veterinary Pathology,[1] Veterinary Microbiology and Parasitology,[2] and Veterinary Science,[3] School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana 70803

Received 18 September 1995/Returned for modification 11 January 1996/Accepted 19 March 1996

Subcutaneous inoculation of dogs with *Ehrlichia canis* was investigated as a more appropriate model of canine ehrlichiosis, which is naturally transmitted by arthropod vectors. A dose-dependent response occurred following subcutaneous inoculation of seven groups of dogs with low concentrations of *E. canis*-infected canine-origin cells. Ehrlichial infection in dogs was defined as concurrence of an increased titer of anti-*E. canis* immunoglobulin G (IgG) antibody in serum, a decreased platelet concentration, and isolation of *E. canis* by blood culture. In dogs administered the two lowest doses, no changes were detected. In seven of nine dogs administered three intermediate doses, the only change detected was a transient and mild increase in the anti-*E. canis* IgG antibody titer in serum. Only two of nine dogs inoculated with the intermediate doses developed an ehrlichial infection. Five of six dogs administered the two highest doses of *E. canis* developed an ehrlichial infection. These dogs had the highest IgG antibody titers in serum and the earliest isolation of *E. canis* from blood. In dogs that developed an ehrlichial infection, thrombocytopenia occurred by 28 days after inoculation, while increased IgG antibody titers in serum and blood cultures positive for *E. canis* occurred as early as 14 days postinoculation. Thrombocytopenia and seroconversion occurred later in the course of infection than previously reported for ehrlichial infections induced by intravenous inoculation. The route of administration and *E. canis* inoculum size can influence the course of ehrlichial infection and should be regarded as important variables in experimentally induced canine ehrlichiosis.

Acute infection with *Ehrlichia canis* occurs in dogs throughout the world and is characterized by fever, thrombocytopenia, proteinuria, and hypergammaglobulinemia (7, 11). Infection of animals other than dogs has not been documented for *E. canis*. Experimentally induced ehrlichial infections in dogs are an important tool in the elucidation of the pathogenesis and evaluation of diagnostic tests (1, 2, 5, 10). In these previously reported studies, *E. canis* infection was induced in dogs by intravenous inoculation by using either infective blood from dogs with acute ehrlichiosis or *E. canis*-infected cell cultures.

In its natural occurrence, *E. canis* infection is transmitted to dogs by *Rhipicephalus sanguineous* (4). Therefore, the intradermal or subcutaneous route of inoculation with a low concentration of infective ehrlichiae likely represents a more appropriate model of ehrlichial infection. Experimental ehrlichial infections that reflect the naturally acquired ehrlichiosis of dogs would permit more reliable determination of diagnostic test sensitivity, as well as the efficacy of treatment and prevention regimens. The objectives of this study were to investigate acute *E. canis* infections induced by subcutaneous inoculations and compare the effects of different doses of infective ehrlichiae. In addition, cell culture methods were utilized to determine the relative concentration of infective ehrlichiae in the inoculum and to detect ehrlichemia in infected dogs.

MATERIALS AND METHODS

Experimental animals. One-year-old male healthy beagles obtained from a commercial source (Liberty Laboratories, Waverly, N.Y.) were housed in individual runs at the facilities of Louisiana State University Laboratory Animal Resources, which are accredited by the American Association for the Accreditation of Laboratory Animal Care. Prior to study, dogs were demonstrated to lack abnormalities on physical examination and complete blood counts and to have no detectable antibodies to *E. canis* in serum by an immunofluorescent-antibody test. The dogs had received routine veterinary medical care prior to this study. The experimental protocol was approved by the Laboratory Animal Care and Use Committee at Louisiana State University.

Experimental design. Seven groups of three dogs each were inoculated with a 0.01-, 0.05-, 0.1-, 0.25-, 0.5-, 1.0-, or 2.0-ml aliquot of an ehrlichial inoculum. Each dose was brought to a 2.0-ml final volume with physiologic buffered saline (PBS) and injected subcutaneously into dogs on the dorsal midline between scapulae. The following parameters were measured at 7, 14, 21, 28, and 35 days postinoculation (dpi): platelet concentration, anti-*E. canis* immunoglobulin G (IgG) antibody titer in serum, and blood culture for *E. canis*. Dogs with ehrlichial infections were identified by concurrence of increased IgG antibody titers in serum, decreased platelet concentrations ($<200 \times 10^3/\mu l$), and isolation of *E. canis* from blood. Dogs that developed ehrlichial infections during the study were treated with 10 mg of doxycycline per kg for 10 days.

Inoculum. A Louisiana isolate of *E. canis* was propagated in monolayers of canine-origin cells with Fischer's medium (GIBCO, Grand Island, N.Y.) containing 20% horse serum (Hyclone, Logan, Utah), 0.2% hydrocortisone (Sigma, St. Louis, Mo.), 2 mM L-glutamine (GIBCO), and no antibiotics in tightly capped culture flasks at 37°C with 5% CO_2. The canine-origin cells have not been completely characterized but are phenotypically and functionally similar to mac-

TABLE 1. *E. canis* inoculum infectivity following serial dilution and passage of 1-ml aliquots to noninfected canine-origin cell cultures[a]

Dilution	Assay result obtained with cells stored:		
	Nonfrozen	In liquid nitrogen without DMSO	In liquid nitrogen with 10% DMSO
Undiluted	Positive	Positive	Positive
10^{-1}	Positive	Positive	Positive
10^{-2}	Positive	Negative	Positive
10^{-3}	Positive	Negative	Positive
10^{-4}	Positive	Negative	Negative
10^{-5}	Negative	Negative	Negative
10^{-6}	Negative	Negative	Negative

[a] The ehrlichial inoculum was assayed before and after storage in liquid nitrogen for 7 days, with or without addition of 10% DMSO. Positive or negative infectivity was determined after 14 days of incubation of cell cultures by immunofluorescence detection with an indirect fluorescent-antibody assay for *E. canis*.

* Corresponding author. Phone: (504) 346-3219. Fax: (504) 346-3226.

TABLE 2. Blood platelet concentrations and serum anti-*E. canis* IgG antibody titers in dogs inoculated with *E. canis* subcutaneously[a]

Time (dpi)	A1		A2		A3		B1		B2		B3		C1		C2		C3		D1	
	PLT[b]	IFA[c]	PLT	IFA	PLT	IFA	PLT	IFA	PLT	IFA	PLT	IFA	PLT	IFA	PLT	IFA	PLT	IFA	PLT	IFA
0	321	Neg[d]	329	Neg	327	Neg	384	Neg	369	Neg	337	Neg	363	Neg	379	Neg	300	Neg	345	Neg
7	292	Neg	280	Neg	244	Neg	337	Neg	380	Neg	277	Neg	357	Neg	326	Neg	313	Neg	287	Neg
14	254	Neg	300	Neg	308	Neg	390	Neg	424	Neg	331	Neg	372	40	348	Neg	367	Neg	301	40
21	264	Neg	236	Neg	319	Neg	331	Neg	358	Neg	322	Neg	318	Neg	324	Neg	364	Neg	305	40
28	328	Neg	375	Neg	378	Neg	420	Neg	390	Neg	339	Neg	71	160	322	Neg	344	40	343	Neg
35	288	Neg	371	Neg	371	Neg	395	Neg	372	Neg	403	Neg	38	160	422	Neg	346	80	373	Neg

[a] Ehrlichial inoculum volumes: group A, 0.01 ml; group B, 0.05 ml; group C, 0.1 ml; group D, 0.5 ml; group E, 1.0 ml, group F, 2.0 ml.
[b] PLT, blood platelet concentration ($10^3/\mu l$).
[c] IFA, serum anti-*E. canis* IgG antibody titer determined by immunofluorescent-antibody assay.
[d] Neg, negative.

rophages and originated from a primary culture of canine bone marrow cells. A single pool of highly infected cells (greater than 90% of cells with *E. canis* immunoreactivity demonstrated by indirect fluorescent-antibody staining) and supernatant medium was collected by scraping flasks 6 days after *E. canis* had been inoculated into the cell culture monolayer. The concentration of intact cells in the resulting suspension was 7×10^5/ml, as counted with hematocytometer. The pooled suspension was aliquoted into 1-ml vials and placed directly into liquid nitrogen storage without cryopreservative. These aliquots were maintained in liquid nitrogen for 7 days prior to inoculation of dogs and cell cultures.

Hematology. Two milliliters of venous blood was collected from the jugular veins of dogs and placed in tripotassium EDTA-containing tubes (Becton-Dickinson, Rutherford, N.J.). Platelet concentrations were determined with a multichannel electronic cell counter (System 9000; Serono-Baker, Allentown, Pa.) validated for canine blood cells. Wright-stained blood smears were also examined to rule out platelet clumping as an artifactual cause of decreased platelet concentrations.

Fluorescent-antibody detection of IgG antibody in serum and immunoreactivity to *E. canis*. For immunofluorescent-antibody testing, serum samples were serially diluted twofold with PBS and tested to the highest negative titer, beginning with a 1:40 dilution. A 50-μl volume of diluted serum was added to a cold acetone-fixed drop suspension of *E. canis*-infected canine-origin cells, incubated for 30 min at 37°C, and then rinsed twice with PBS. A 50-μl volume of a 1:80 dilution of fluorescein-conjugated goat anti-dog IgG (Kirkegaard & Perry, Gaithersburg, Md.) was added to a slide and incubated for 30 min at 37°C. The slide was rinsed twice with PBS, and immunofluorescence was detected with a fluorescence microscope. Test controls included seropositive canine serum, seronegative canine serum, and noninfected canine-origin cell line.

For detection of *E. canis* immunoreactivity in infected cell cultures by fluorescent-antibody testing, 50 μl of seropositive canine serum diluted 1:40 with PBS was added to cold acetone-fixed cytocentrifuged smears of supernatant medium from cell cultures, incubated for 30 min at 37°C, and then rinsed twice with PBS. A 50-μl volume of fluorescein-conjugated goat anti-dog IgG diluted 1:80 in PBS was added, and the mixture was incubated for 30 min at 37°C, rinsed twice with PBS, and examined for fluorescence with a fluorescence microscope.

Blood culture for *E. canis*. Five-milliliter samples of blood from jugular veins of dogs were collected in EDTA-containing tubes and centrifuged at $500 \times g$ for 15 min. The buffy coat cells, approximately 1 ml, were removed and placed on 1-day-old cultures of confluent canine-origin cells in Fischer's medium with 20% horse serum, 0.2% hydrocortisone, 2 mM L-glutamine, and no antibiotics. After 24 h of incubation, the supernatant medium with nonadherent cells was discarded and replaced with fresh medium. Full medium changes were made weekly thereafter. Concentrated smears of supernatant medium were prepared with a cytocentrifuge (Cytospin; Shandon, Pittsburg, Pa.) at 7, 14, 21, and 28 days of incubation and stained by fluorescent-antibody testing and Wright's stain for *E. canis* infection.

In vitro titration studies. To assess the infectivity of the ehrlichial inoculum, 1-ml samples of 10-fold serial dilutions (through 10^{-6}) of fresh inoculum, liquid nitrogen frozen without cryopreservative for 7 days, and liquid nitrogen frozen with 10% dimethyl sulfoxide (DMSO) (Sigma Chemical, St. Louis, Mo.) for 7 days were each added to 1-day-old confluent cultures of canine-origin cells with Fischer's medium, 20% horse serum, 2 mM L-glutamine, and 0.2% hydrocortisone at 37°C. After weekly medium changes, cytocentrifuged smears of supernatant medium from each culture flask were prepared after 21 days of incubation and *E. canis* immunoreactivity by FAT and Wright-stained smears were examined to determine the presence of ehrlichial infection.

Statistical analysis. The *t* test was used to detect any significant changes that occurred in platelet concentrations of infected dogs.

RESULTS

The titration assay demonstrated that dilution of *E. canis*-infected cells diminished the in vitro infectivity for canine cell cultures (Table 1). Storage of *E. canis*-infected cells with addition of 10% DMSO for 7 days in liquid nitrogen slightly decreased the in vitro infectivity of the inoculum, while storage in liquid nitrogen without DMSO more markedly decreased the in vitro infectivity of *E. canis* (Table 1).

In the two groups of dogs inoculated with lowest doses (group A, 0.01 ml; group B, 0.05 ml) of the *E. canis* inoculum, no significant changes occurred in blood platelet concentration and anti-*E. canis* IgG antibody titers in serum remained less than 1:40 (Table 2). Blood cultures from these two groups of dogs were negative for ehrlichiae through 35 dpi.

In the intermediate-dosage groups (group C, 0.10 ml; group D, 0.25 ml; group E, 0.5 ml), most dogs had either no change (three [33%] of nine dogs) or a transient increase to 1:40 at 14 and 21 dpi only (four [44%] of nine dogs) in anti-*E. canis* IgG antibody titers in serum (Table 2). In these seven dogs, no significant changes in blood platelet concentrations occurred and all blood cultures for ehrlichiae were negative. Two (22%) of nine dogs, one each from groups C and E, were thrombocytopenic at 28 and 35 dpi, had anti-*E. canis* antibody titers in serum of 1:80 and 1:160 at 35 dpi, and had *E. canis*-positive blood cultures (Table 2).

In the highest-dose groups (group F, 1.0 ml; group G, 2.0 ml), five (83%) of six dogs developed ehrlichial infections, as indicated by thrombocytopenia, increased IgG antibody titers in serum beginning at 14 to 25 dpi, and blood cultures positive for *E. canis* (Tables 2 and 3). These five dogs had the highest IgG antibody titers detected, ranging from 1:320 to 1:1,280 at 35 dpi. In one dog in group F, the only change detected was a transient serum antibody titer increase to 1:40 at 28 dpi (Table 2).

In the seven dogs from all groups that had blood cultures positive for *E. canis* following subcutaneous inoculation, platelet concentrations were significantly lower ($P < 0.03$) (Fig. 1) and IgG antibody titers in serum were greater than those of the other dogs at 21, 28, and 35 dpi.

DISCUSSION

The titrational assay demonstrated that the in vitro infectivity of an *E. canis* inoculum for canine-origin cell cultures decreased following dilution. While immunofluorescent-antibody testing and PCR techniques can assess the ehrlichial concentration of infected cells, antigenic and nucleic acid markers of ehrlichiae do not reflect ehrlichial viability or infectivity. Therefore, a cell culture assay such as this is indicated for determination of the ehrlichial infectivity of inoculums. The titration assay also indicated a marked decrease in ehrlichial infectivity following short-term storage in liquid nitrogen without DMSO, compared with nonfrozen aliquots and aliquots

TABLE 2—*Continued*

D2		D3		E1		E2		E3		F1		F2		F3		G1		G2		G3	
PLT	IFA	PLT	IFA	PLT	IFA	PLT	IFA	PLT	IFA	PLT	IFA	PLT	IFA	PLT	IFA	PLT	IFA	PLT	IFA	PLT	IFA
442	Neg	301	Neg	404	Neg	295	Neg	260	Neg	392	Neg	429	Neg	357	Neg	289	Neg	340	Neg	299	Neg
302	Neg	281	Neg	351	Neg	280	Neg	270	Neg	376	Neg	438	Neg	376	Neg	332	Neg	316	Neg	275	Neg
423	Neg	285	40	313	40	282	40	218	Neg	367	Neg	494	Neg	312	Neg	335	160	323	80	309	160
419	Neg	246	40	278	40	256	40	189	Neg	366	Neg	580	Neg	281	40	193	320	254	320	180	80
390	Neg	372	Neg	310	Neg	307	Neg	105	40	271	40	419	40	120	640	89	640	138	160	38	640
342	Neg	300	Neg	372	40	384	Neg	89	80	165	320	414	Neg	53	320	100	1,280	52	320	31	1,280

with 10% DMSO stored in liquid nitrogen. This low-temperature attenuation served to decrease the concentration of infective ehrlichiae in the inoculum further. The concentrations of infective ehrlichiae in the doses administered in this study are likely similar to the single or few ehrlichiae that are transmitted by arthropod vectors. While the number of infective ehrlichiae in this inoculum was not quantitated, it was estimated from the titration assay that the inoculum contained approximately 2×10^4 infective U/ml. The marked inactivation of ehrlichial infectivity at low-temperature storage also indicates that cryopreservatives such as DMSO are needed to maintain maximal infectivity of *E. canis*. Nevertheless, DMSO was not incorporated in the inoculum used in this study because of the potential for adverse effects on dogs and the in vivo infectivity of *E. canis*. Future studies might evaluate whether DMSO has any effects that warrant its removal from an ehrlichial inoculum before administration to animals.

This study demonstrates that induction of *E. canis* infection in dogs with low doses and subcutaneous inoculation follows a dose-dependent pattern. A transient increase in anti-*E. canis* IgG antibody titers in serum was the earliest change observed in dogs inoculated with lower doses. It was not determined whether this antibody response was to antigens of nonviable ehrlichiae or if a limited ehrlichial infection developed that was rapidly cleared. Since weekly blood cultures for ehrlichiae were consistently negative in those dogs with transiently increased serum antibody titers, there is no evidence that ehrlichial infection resulted. Subcutaneous doses of an inoculum that were 5 to 10 times greater than noninfective doses consistently produced prototypical *E. canis* infection in dogs, with marked thrombocytopenia, increased IgG antibody titers in serum, and blood cultures positive for ehrlichiae.

The course of infection following subcutaneous inoculation of *E. canis* differed from that in studies of canine ehrlichiosis that utilized intravenous inoculations. In previous reports, intravenous administration of *E. canis* caused marked thrombocytopenia that occurred at 10 to 14 dpi (1, 2, 5, 8–10). In this study, subcutaneous inoculation induced a marked thrombocytopenia that occurred consistently at 28 dpi. Similarly, seroconversion to *E. canis* reported in studies using intravenous inoculation occurred at 7 to 14 days (12), while dogs with ehrlichial infection after subcutaneous inoculation had increases in serum antibody titers beginning at 14 to 28 dpi. These differences cannot necessarily be attributed to the *E. canis* isolates utilized in this experimental infection, since our previous studies using intravenous administration of this Louisiana isolate also demonstrated onset of seroconversion and thrombocytopenia at 7 to 14 dpi (3). This study did not clarify whether the longer prodromal period with low-dose subcutaneous inoculation resulted from prolonged ehrlichia replication period until a critical pathogenetic concentration was reached because of the initially low concentration of ehrlichiae, or if subcutaneous placement affects pathogenesis by making the liver, spleen, and bone marrow, the presumptive target tissues of *E. canis*, less accessible to ehrlichiae. These results demonstrate that perhaps both the route and dosage used for ehrlichial inoculation affect the severity and onset of the experimental disease. Methods are needed to quantitate the concentration of infective ehrlichiae so that the dosages used in experimental ehrlichial infections can be standardized and compared to those of naturally occurring infections.

An ehrlichial culture system using buffy coat blood cells overlaid on canine-origin cells detected ehrlichemia in all dogs that developed clinical infections. Ehrlichial infection was initially detected in blood collected from dogs at 14 to 28 dpi, although longer incubation times of positive cultures were re-

TABLE 3. Isolation of *E. canis* from blood of 7 of 18 dogs following subcutaneous inoculation with *E. canis*[a]

Time (dpi)	Result for dog:						
	G3	G2	G1	F1	F2	E3	C1
7	Neg	Neg	Neg	Neg	Neg	Neg	Neg
14	28	28	28	Neg	Neg	Neg	Neg
21	28	Neg	21	Neg	Neg	14	14
28	14	14	7	Neg	Neg	21	28
35	7	7	7	21	14	14	14

[a] Buffy coat blood cells from inoculated dogs were added to canine-origin cell cultures that were assayed by indirect fluorescent-antibody assay for *E. canis* infectivity for 28 days at 7-day intervals. Each value is the earliest incubation time (days) at which ehrlichial immunofluorescence was detected in cultures. Neg indicates that no ehrlichial immunoreactivity was detected. After inoculation of dogs with *E. canis*, blood was collected at weekly intervals through 35 dpi.

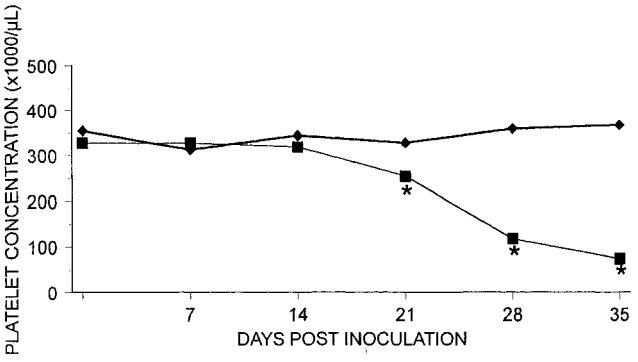

FIG. 1. Changes in platelet concentrations of dogs following subcutaneous inoculation with low doses of *E. canis*. The infected group (■) consisted of 7 dogs with positive isolation of *E. canis* on culture of buffy coat cells; the noninfected group (◆) consisted of 14 dogs that were negative for ehrlichial isolation on buffy coat cultures. Significant differences between the groups are indicated by asterisks.

quired in the early phase of infection to demonstrate infectivity. As the ehrlichial infection progressed, cultures of blood collected from dogs at 28 and 35 dpi were often positive for ehrlichial immunoreactivity after only 7 days of incubation, which is indicative of higher levels of ehrlichemia.

The method of blood culture used for detection of ehrlichial infection in this study is similar in some respects to another recently described method (4, 6). However, that method utilized 30 ml of blood, from which the mononuclear cell fraction of leukocytes was separated by density gradient centrifugation and added to a confluent monolayer of DH82 canine macrophage cells. Our method used buffy coat cells from only 5 ml of blood, which were added directly to a cell culture. In addition, no antibiotics were included in any of the media used in our culture system and fluorescent-antibody testing was used to detect *E. canis* infection of cultures. The other method included antibiotics in the wash medium, and positive cultures were detected by a modified Wright stain for the presence of ehrlichial morulae (4, 6). Without a direct comparative study, it is difficult to compare the sensitivities of these two blood culture methods because of differences between blood culture methods and experimental infections. Nevertheless, the earliest that *E. canis* was isolated from blood of infected dogs by their method was after 14 days of incubation (4), while infectivity was detected by blood culture as early as 7 days of incubation in infected dogs in this study.

In our study, detection of ehrlichemia by blood culture preceded a significant decrease in platelet concentration and paralleled the onset of increased serum antibody titers. In a previous report (4), blood cultures positive for *E. canis* occurred at approximately the same time as decreased platelet concentrations and increased serum antibody titers. However, in that study, ehrlichial infections were induced in German shepherd dogs, some which were thrombocytopenic (platelets, $<200 \times 10^3/\mu l$) prior to inoculation, by intravenous administration of ehrlichiae, and changes in antibody titers and platelets occurred after only 2 days. Results of our low-dose, subcutaneous inoculation study suggest that ehrlichemia and increased antibody titers may precede the occurrence of thrombocytopenia in the natural course of acute *E. canis* infection.

ACKNOWLEDGMENTS

We thank Carol Rinehart and Paul Hine of Mallinckrodt Veterinary, Inc., Mundelein, Ill., for their suggestions in planning this study.

This research was supported by funding provided by Mallinckrodt Veterinary, Inc.

REFERENCES

1. **Buhles, W. C., D. L. Huxsoll, and M. Ristic.** 1974. Tropical canine pancytopenia: clinical, hematologic, and serologic response of dogs to *Ehrlichia canis* infection, tetracycline therapy, and challenge inoculation. J. Infect. Dis. **130:** 357–367.
2. **Codner, E. C., and W. R. Maslin.** 1992. Investigation of renal protein loss in dogs with acute experimentally induced *Ehrlichia canis* infection. Am. J. Vet. Res. **53:**294–299.
3. **Gaunt, S. D., D. C. Baker, R. E. Corstvet, and R. M. Simpson.** 1986. Platelet concentration and platelet aggregation in dogs with experimental *Ehrlichia platys* or *Ehrlichia canis* infection, abstr. 307, p. 54. *In* Abstracts of the Conference of Research Workers in Animal Diseases.
4. **Groves, M. G., G. L. Dennis, H. L. Amyx, and D. L. Huxsoll.** 1975. Transmission of *Ehrlichia canis* to dogs by ticks (*Rhipicephalus sanguineus*). Am. J. Vet. Res. **36:**937–940.
5. **Iqbal, Z., W. Chaichanasiriwithaya, and Y. Rikihisa.** 1994. Comparison of PCR with other tests for early diagnosis of canine ehrlichosis. J. Clin. Microbiol. **32:**1658–1662.
6. **Iqbal, Z., and Y. Rikihisa.** 1994. Reisolation of *Ehrlichia canis* from blood and tissues of dogs after doxycycline treatment. J. Clin. Microbiol. **32:**1644–1649.
7. **Kuehn, N. F., and S. D. Gaunt.** 1985. Clinical and hematologic findings in canine ehrlichiosis. J. Am. Vet. Med. Assoc. **186:**355–358.
8. **Lovering, S. L., K. R. Pierce, and L. G. Adams.** 1980. Serum complement and blood platelet adhesiveness in acute canine ehrlichiosis. Am. J. Vet. Res. **41:**1266–1271.
9. **Pierce, K. R., G. E. Marrs, and D. Hightower.** 1977. Acute canine ehrlichiosis: platelet survival and Factor 3 assay. Am. J. Vet. Res. **38:**1821–1825.
10. **Reardon, M. J., and K. R. Pierce.** 1981. Acute experimental canine ehrlichiosis: sequential reaction of the hemic and lymphoreticular systems. Vet. Pathol. **18:**48–61.
11. **Troy, G. C., and S. D. Forrester.** 1990. Canine ehrlichiosis, p. 404–414. *In* C. Greene (ed.), Infectious diseases of the dog and cat. The W. B. Saunders Co., Philadelphia.
12. **Weisiger, R. M., M. Ristic, and D. L. Huxsoll.** 1975. Kinetics of antibody response to *Ehrlichia canis* assayed by the indirect fluorescent antibody method. Am. J. Vet. Res. **36:**689–694.