

Analyses of *Ehrlichia canis* and a Canine Granulocytic *Ehrlichia* Infection

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Ehrlichia canis and canine granulocytic *Ehrlichia* sp. (CGE) infect canine monocytes and granulocytes, respectively. *E. canis* has been cultured in vitro and used to develop an immunofluorescence assay. CGE has not been cultured, and a serologic assay is not available. The sera of dogs infected with CGE were reported to react with *E. canis* by immunofluorescence. In this study, the temporal response of immunoglobulin G (IgG) was determined by an enzyme-linked immunosorbent assay (ELISA) with purified *E. canis* antigen in four dogs experimentally infected with *E. canis*, in two dogs experimentally infected with CGE, and in one dog infected with *E. canis* and subsequently infected with CGE. *E. canis*-infected dogs developed an IgG ELISA result of 1.5 or greater for the optical density signal/noise ratio by 2 months postinfection. CGE challenge of a dog with a previous *E. canis* infection induced an anamnestic increase in the IgG ELISA result; however, CGE infection alone did not induce a significant IgG ELISA response. Western immunoblot analysis showed that dogs infected with *E. canis* developed antibodies initially that reacted with low-molecular-mass proteins (30, 24, and 21 kDa) and subsequently with higher-molecular-mass proteins (160, 100, 78, 74, 64, 47, and 40 kDa). In contrast, CGE-infected dogs showed reactions with the same higher-molecular-mass proteins of *E. canis* but, unlike *E. canis*-infected dogs, not with the low-molecular-mass proteins of *E. canis*. Of 10 serum samples collected in the field in Indonesia from dogs with tropical canine pancytopenia, all had an optical density signal minus noise value of 2.54 or greater in the IgG ELISA and reacted with *E. canis* antigen in a pattern similar to that of serum samples from dogs experimentally infected with *E. canis* in Western immunoblotting. This study suggests that the IgG ELISA and Western immunoblotting with purified *E. canis* as the antigen are useful in distinguishing between *E. canis* and CGE infections in dogs.

Canine ehrlichiosis (tropical canine pancytopenia) was originally described in Algeria in 1935 (7) and soon after in Africa, the Middle East, and the Orient (9). The clinical disease was recently recognized in Indonesia (19). The disease, caused by a rickettsial organism, *Ehrlichia canis*, is transmitted by ticks. Ehrlichiosis was recognized in dogs in the United States in 1962 (8) but is now known throughout much of the world. During the Vietnam War, 160 U.S. military dogs died from *E. canis* infections (20).

Canine ehrlichiosis exhibits acute, subclinical, and chronic phases. In the acute phase, clinical signs, such as fever, depression, dyspnea, anorexia, lymphadenopathy, and slight weight loss, are observed. The chronic phase is characterized by hemorrhages, epistaxis, peripheral edema, emaciation, and hypotensive shock, leading to death. Laboratory findings include thrombocytopenia, leukopenia, and hypergammaglobulinemia (3, 12). *E. canis* infects peripheral blood monocytes and can be isolated from these cells (15).

Canine granulocytic ehrlichiosis is a milder disease than classical canine ehrlichiosis and is sometimes associated with polyarthritis (2, 4, 5, 10, 11), whereas monocytic *E. canis* infection is not. Canine granulocytic *Ehrlichia* sp. (CGE) and *E. canis* are antigenically cross-reactive to a limited degree but apparently are not cross-protective (10).

Laboratory diagnosis of canine leukocytic ehrlichiosis is made serologically and by observation of organisms in Romanovsky-stained peripheral blood or buffy coat smears (12). The indirect fluorescent-antibody test (IFA) with *E. canis* cultured in primary canine blood monocytes (18), in DH82 cells (6), or in "spontaneously immortalized" mouse peritoneal macrophage-dog blood monocyte hybrid MDH-SP cells (13) as the antigen has been used for the serologic diagnosis of canine ehrlichiosis. Success in continuous culturing of *E. canis* in a canine macrophage cell line (6) allowed the production of *E. canis* organisms in large quantities sufficient for purification. CGE affects granulocytes and has not been cultured in vitro, preventing the development of specific serologic assays for this organism. The purpose of this study was to compare the temporal development of immunoglobulin G (IgG) antibodies in *E. canis*- and CGE-infected dogs by using an enzyme-linked immunosorbent assay (ELISA) and Western blot (immunoblot) analysis with purified *E. canis* as the antigen.

MATERIALS AND METHODS

Culturing and purification of *E. canis*. *E. canis* isolated from the blood of an experimentally infected dog was cultured in dog macrophage cell line DH82 as previously described (6). *E. canis* was purified from between 5 and 10

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150-cm² flasks of infected DH82 cells as previously described (17).

Experimental infection of dogs. One conditioned German shepherd was purchased from Butler, Wolcott, N.Y. The dog was intravenously inoculated with 10⁷ *E. canis*-infected DH82 cells. Prednisone was orally administered daily at 2 to 4 mg/kg from day 118 to day 145 postinfection. A second challenge of 10⁷ *E. canis*-infected DH82 cells was given on day 149 postinfection. Rectal temperature, appetite, attitude, and other abnormalities were monitored daily. Blood (20 ml) was collected weekly to obtain serum and for leukocyte and thrombocyte counts. Buffy coat smears were stained with Diff-Quik (Baxter Scientific Co., Obetz, Ohio) and examined for the presence of *E. canis*. Buffy coat fractions derived from 15 ml of heparinized blood were overlaid on DH82 cell monolayers and cultured to determine how long it would take to isolate *E. canis* from blood (6).

Six dogs were purchased locally in Oklahoma. One dog was infected with *E. canis* by allowing experimentally infected ticks (*Dermacentor variabilis*) detached from a carrier dog to feed on it. Three dogs were infected by intravenous inoculation of 8 to 10 ml of heparinized blood from dogs that were carriers of *E. canis*. Two dogs were infected with CGE by inoculation of whole blood from a carrier. One dog infected with *E. canis* by whole-blood inoculation was challenge exposed to CGE on day 78 following the initial exposure to *E. canis*. Blood smears were examined daily for the presence of parasites; sera were collected at 1- to 2-week intervals to measure antibody levels.

Field-case serum samples. Serum samples from 10 dogs with a clinical disease symptomatically compatible with tropical canine pancytopenia were collected at the College of Veterinary Medicine, Institute Pertanian Bogor, Bogor, Indonesia. Serum samples were also collected from three blood donor dogs and seven dogs without clinical signs of canine ehrlichiosis at the Veterinary Teaching Hospital, College of Veterinary Medicine, The Ohio State University, Columbus, to serve as negative controls.

ELISA. An indirect ELISA was used to detect and quantify IgG to *E. canis* in the test sera. ELISA 96-well microtiter plates (Flow Laboratories, Inc., McLean, Va.) were coated with the purified ehrlichial antigen and the control antigen (DH82 cell sonic homogenate supernatant) at 2 µg of protein per well in alternating columns as described previously (16). The plates were blocked with a 5% (wt/vol) nonfat dry milk (Carnation Co., Los Angeles, Calif.) solution in phosphate-buffered saline (PBS). The ELISA was performed by adding 0.1 ml of a 1:100 dilution of test sera (diluted in 5% milk-PBS) to both ehrlichial and control antigen wells and by incubating the plates at 37°C for 1 h. Three successive rinses of wells with PBS-Tween 20 were followed by the addition of 0.1 ml of horseradish peroxidase-conjugated goat anti-dog IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.), diluted 1:1,000 in 5% milk-PBS, to each well, and the plates were incubated and rinsed as described above (16). The substrate [0.015% 2,2'-azino-bis(3-ethylbenzthiazoline sulfonic acid)diammonium salt (Sigma Chemical Co., St. Louis, Mo.)-0.03% hydrogen peroxide in 0.1 M citrate buffer (pH 4.5)] was added to each well. After 10 min in darkness at 25°C, the optical density of each well was measured at 405 nm with a UV max ELISA plate reader (Molecular Devices Corp., Menlo Park, Calif.). The signal/noise (S/N) ratio and the signal minus noise (S - N) value for each test sample were determined by dividing and subtracting, respectively, the optical density of the ehrlichial antigen well by that of the control antigen well.

Serum samples were tested in triplicate; standard deviations for mean optical densities were 0 to 5% (average, 1.6%). Means for each test sample were reported. Within-test variability was approximately 25% when different batches of *E. canis* antigen were used.

IFA. The IFA was performed as previously described (16). In brief, *E. canis*-infected cells suspended in culture medium were air dried and acetone fixed on 12-well Teflon (E.I. DuPont de Nemours & Co., Inc., Wilmington, Del.)-coated multiwell slides (Cell-Line Associates, Newfield, N.J.). Serial twofold dilutions of test sera in PBS, starting at a 1:20 dilution, were placed in 10-µl quantities in the wells of antigen-coated IFA slides. The slides were incubated in a humidified 37°C incubator for 30 min. The slides were rinsed three times in PBS containing 0.002% Tween 20, and 10 µl of fluorescein-conjugated goat anti-dog IgG (United States Biochemical Corp., Cleveland, Ohio), diluted 1:200 in PBS, was added to each well. The slides were incubated, washed, and blotted as previously described (16) and observed with a fluorescence microscope.

Western blot analysis. Purified *E. canis* antigens were separated by sodium dodecyl sulfate (SDS)-10 to 20% polyacrylamide gradient or SDS-12.5% polyacrylamide gel electrophoresis, and Western blotting was performed with various dog sera and alkaline phosphatase-conjugated affinity-purified anti-dog IgG as previously described (17). An uninfected DH82 cell extract was used as the control antigen.

Ehrlichia risticii, *Ehrlichia sennetsu*, and *Neorickettsia helminthoeca* were cultured and antigens were purified as previously described (17). These antigens were separated by SDS (4 to 20% gradient) gel electrophoresis, and Western blotting was performed with various canine sera. Antisera against *N. helminthoeca* were obtained from experimentally infected dogs (17). These dogs were seronegative against *E. canis*, *E. risticii*, *E. sennetsu*, and *N. helminthoeca* before infection with *N. helminthoeca* (16).

RESULTS

A dog inoculated with cultured *E. canis* developed transient fever (39.5 to 40.8°C) and thrombocytopenia (platelet count of 30 × 10⁹ to 60 × 10⁹/liter) accompanied by mild depression and anorexia on day 2; the symptoms lasted approximately 1 month. There were no clinical signs or hematologic changes after the second inoculation. Parasitemia became apparent in buffy coat smears starting on day 10 and lasting up to day 20. Prednisone treatment induced parasitemia (Fig. 1A) but did not induce fever or thrombocytopenia. Although *E. canis* was not easily seen in buffy coat smears, *E. canis* was continuously isolated from the buffy coat fraction after day 20 and up to day 60, after which isolation became sporadic (Fig. 1A). Prednisone treatment shortened the number of culturing days required for isolation (Fig. 1A). All preinfection dog sera were negative at a dilution of 1:20 in the IFA, and the ELISA S/N ratio and S - N value were 1.31 ± 0.30 and 0.16 ± 0.17 (n = 5), respectively. The IFA titer and the ELISA S - N value and S/N ratio continued to rise up to approximately 80 to 90 days postinfection and then declined but remained positive until the termination of the experiment (Fig. 1B and C). The maximum S/N ratio was 8.18. Following prednisone treatment, the IFA titer declined as much as eightfold and the ELISA results declined slightly (Fig. 1B and C).

ELISA results were similar for the remaining dogs experimentally infected with *E. canis*. The IgG ELISA value rose

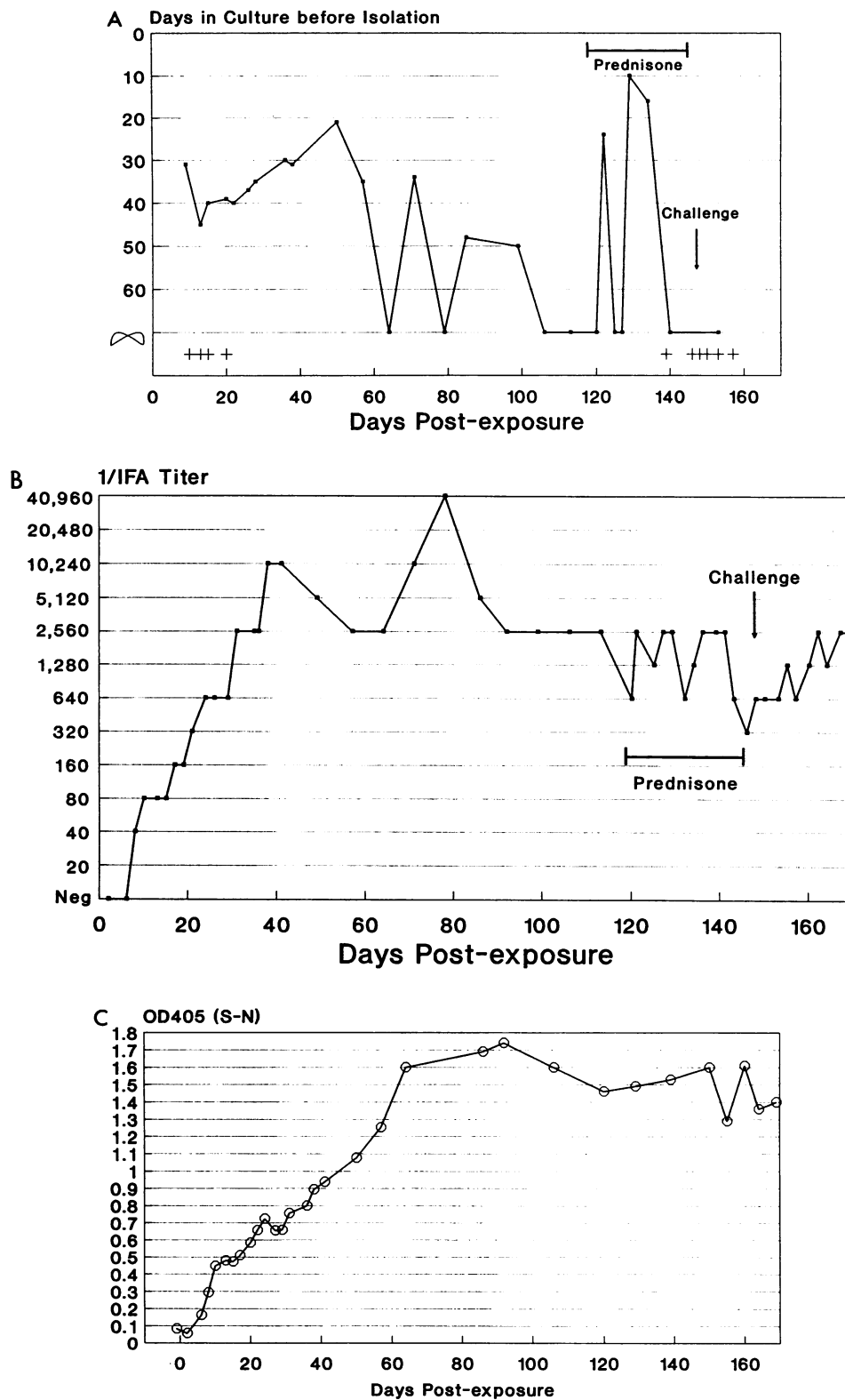


FIG. 1. Parasitemia, IgG IFA, and IgG ELISA results in a dog infected with cell-cultured *E. canis*. (A) *E. canis* isolation from the peripheral blood of a dog infected with *E. canis*. Blood was collected once or twice every week, and monocyte fractions were overlaid on DH82 cell monolayers. The infectivity of cultured cells was evaluated every other day after staining of cytospin-prepared cells with Diff-Quik. For each blood monocyte culture from the dog, the first day on which the culture became clearly positive with *E. canis* was recorded. The culture period (days) required for positive *E. canis* identification for each blood collection is indicated on the vertical axis. The culture was considered negative when no *E. canis* was found after 60 days of culturing. +, *E. canis* found in buffy coat smears. (B) IFA titer. (C) IgG ELISA result (S - N value) for a dog experimentally infected with cell-cultured *E. canis*. OD405, optical density at 405 nm.

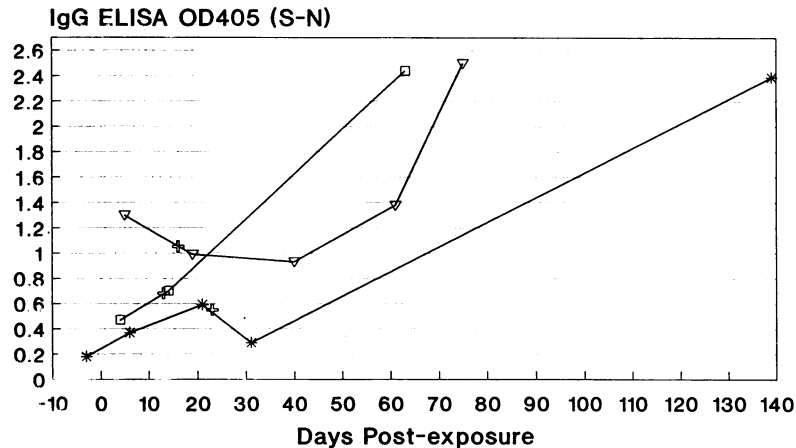


FIG. 2. Response curve of IgG ELISA results (S - N value) for three dogs infected with *E. canis* by whole-blood transfusion (dogs 57 [▽] and 226 [□]) or tick transmission (dog 224 [*]). +, morulae first observed in monocytes. OD405, optical density at 405 nm.

more slowly in the dog infected by tick bite than in other dogs (Fig. 2), and the onset of parasitemia was also slower in this dog (23 days) than in dogs infected by whole-blood transfusion (13 to 16 days) or by injection of cell-cultured *E. canis* (10 days).

The dog inoculated with *E. canis* developed an IgG ELISA anamnestic response when challenged with CGE (Fig. 3). *E. canis* was found in monocytes before and after challenge with CGE. CGE organisms were found in the peripheral blood neutrophils 50 days after challenge. IgG ELISA results against *E. canis* in two dogs infected with CGE were not significantly elevated. The highest IgG ELISA result occurred at day 70 postinoculation in one dog (S - N value = 0.14 ± 0.03 ; S/N ratio = 1.19 ± 0.05 ; $n = 3$ independent assays) and at day 103 postinoculation in another (S - N value = 0.18 ± 0.05 ; S/N ratio = 1.13 ± 0.04 ; $n = 3$ independent assays).

All Indonesian dogs were found strongly seropositive by both the IgG IFA and the IgG ELISA (Table 1). All blood donor or control dogs tested were found seronegative at a titer of 1:20 by the IgG IFA, and the S - N value and S/N ratio in the IgG ELISA were 0.70 ± 0.24 and 1.53 ± 0.03 , respectively.

A Western immunoblot analysis of the temporal develop-

ment of antibodies against *E. canis* antigen is shown in Fig. 4. A dog experimentally infected with *E. canis* developed antibodies which reacted with low-molecular-mass polypeptides (30, 24, and 21 kDa) of *E. canis* at day 10 postexposure and which progressively responded to higher-molecular-mass polypeptides (160, 100, 78, 74, 64, 47, and 40 kDa) later in infection (Fig. 4). At day 50 postinfection, canine anti-CGE sera produced binding patterns similar to those of anti-*E. canis* sera with 78-, 74-, 64-, 47-, 46-, 44-, and 40-kDa antigens but showed a minimum reaction to no reaction with the dominant *E. canis* antigens, 30, 24, and 21 kDa (Fig. 4 and 5).

Serum samples from 10 dogs diagnosed with naturally occurring tropical canine pancytopenia in Indonesia reacted in almost identical patterns with purified *E. canis* antigen (only 2 of the 10 serum samples tested are shown in Fig. 5). Sera from experimental *E. canis* infections produced by three different methods reacted with the polypeptides of *E. canis* (78-, 74-, 64-, 47-, 44-, 41-, 40-, 30-, 27-, 25-, 24-, 21-kDa) in a manner similar to that of sera from dogs naturally infected in Indonesia (Fig. 5). None of these sera reacted with antigens obtained from uninfected DH82 cells (Fig. 5). As reported previously (17), anti-*N. helminthoeca* serum strongly reacted with 78- and 64-kDa bands of *E.*

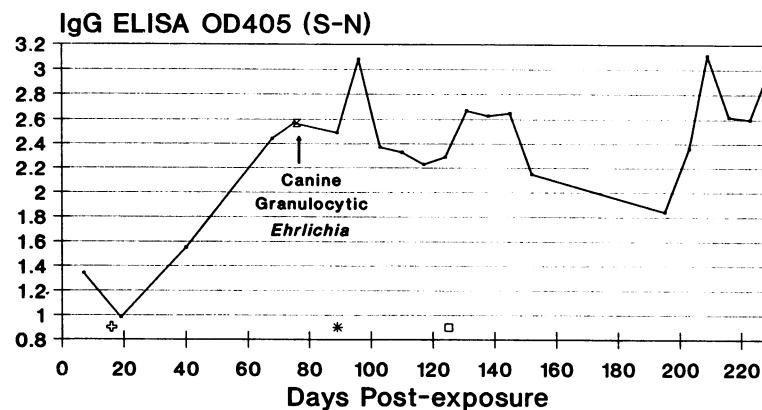


FIG. 3. Response curve of IgG ELISA results (S - N value) a dog infected with *E. canis* by whole-blood transfusion and then challenge exposed to CGE by whole-blood transfusion. +, morulae first observed in monocytes; *, recurrence; □, morulae first observed in neutrophils. OD405, optical density at 405 nm.

TABLE 1. ELISA and IFA results for IgG antibodies to *E. canis* in serum samples from dogs with clinical signs of tropical canine pancytopenia in Indonesia

Dog	IFA titer	ELISA result ^a	
		S - N value	S/N ratio
H	10,240	2.60	2.95
H1	10,240	2.94	2.94
H2	5,120	3.08	3.88
H4	5,120	2.54	2.64
H6	2,560	2.68	2.70
H7	10,240	2.71	2.83
H8	10,240	3.01	3.53
H9	5,120	3.08	4.24
G12	1,280	3.25	4.41
G18	2,560	2.60	2.65

^a For control sera, the S - N value = 0.70 ± 0.24 (n = 10) and the S/N ratio = 1.53 ± 0.03 (n = 10).

canis (Fig. 4). When tested with three ehrlichial antigens and one neorickettsial antigen, CGE antisera showed significantly reacting bands only with *E. canis* antigen and not with *N. helminthoeca*, *E. sennetsu*, or *E. risticii* antigen (data not shown), like *E. canis* antisera, as shown previously (17).

DISCUSSION

Like the IFA, the ELISA is useful in detecting *E. canis* antibodies. Some differences in the response curves observed with the IFA and ELISA may result in part from different preparations of antigens, i.e., acetone-fixed whole cells versus soluble components that bind to ELISA wells. The reason for the slow IgG ELISA response in dogs

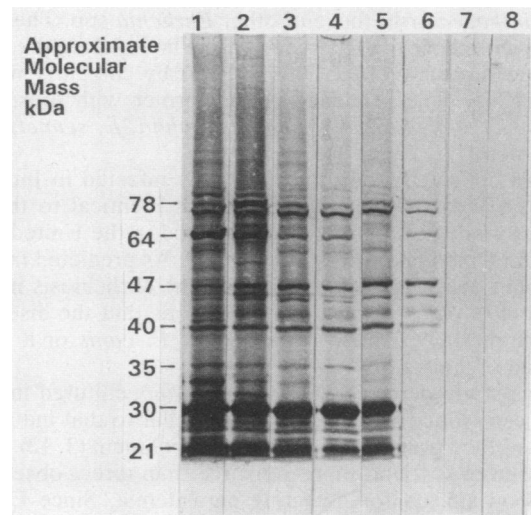


FIG. 5. Western blot analysis of various sera against the *E. canis* antigen (except for lanes 7 and 8) separated by SDS-12.5% polyacrylamide gel electrophoresis. IgG IFA titers or ELISA results for these antisera are shown in Fig. 1 to 3 and Table 1. Lanes (antisera): 1, dog H, chronic *E. canis* field infection in Indonesia, duckhound; 2, dog H1, chronic *E. canis* field infection in Indonesia, German shepherd; 3, dog 226, experimental *E. canis* infection, day 65 postinfection by transfusion of whole blood from a carrier dog; 4, dog 224, experimental *E. canis* infection, day 140 postinfection after infected ticks were allowed to feed on the dog; 5, dog 1, experimental *E. canis* infection with cell-cultured *E. canis*, day 79 postinfection; 6, dog 1090, experimental CGE infection by whole-blood transfusion, day 70 postinfection; 7, antiserum same as that in lane 5, antigen = uninfected DH82 cells; 8, antiserum same as that in lane 6, antigen = uninfected DH82 cells.

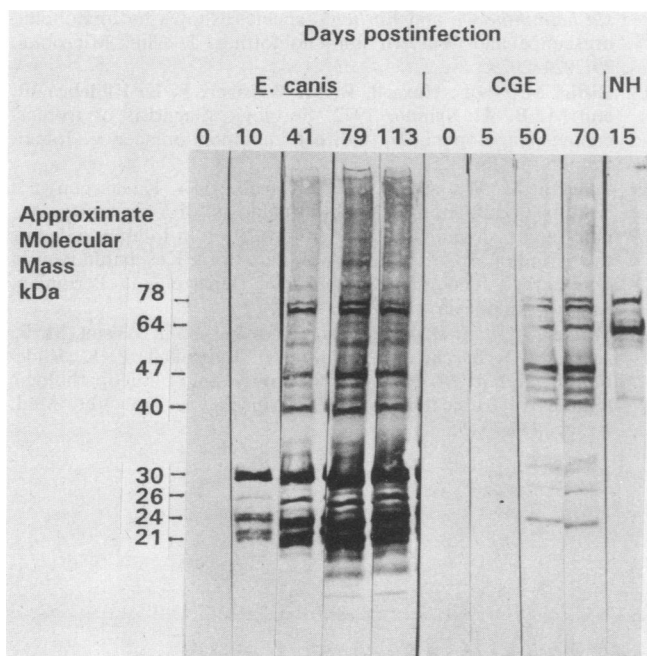


FIG. 4. Western blot analysis with purified *E. canis* as the antigen of sera collected consecutively from a dog experimentally infected with *E. canis* cultured in DH82 cells, from a dog infected with CGE, and from a dog infected with *N. helminthoeca* (NH). SDS-10 to 20% polyacrylamide gradient gel.

infected by tick attachment is unknown, but it may be a dose-related phenomenon. Furthermore, in tick transmission, it is not known precisely when transmission occurs after tick attachment.

Since in the IgG ELISA sera from CGE-infected dogs did not develop any significant levels of antibodies to *E. canis*, the IgG ELISA most likely detects *E. canis*-specific antigens and not *Ehrlichia* common antigens. Since *E. canis* and CGE are the closest to each other of all the *Ehrlichia* spp. examined except the human ehrlichiosis agent (1), the *E. canis* IgG ELISA would show the least cross-reaction with antisera to other ehrlichial species. In contrast, Western blotting revealed both common and specific antigens between *E. canis* and CGE. There were few or no reacting antibodies to polypeptides at molecular masses lower than 30 kDa in the CGE infection. On the contrary, there was a stronger reaction to low- than to high-molecular-mass polypeptides in the *E. canis* infection, especially at the early stages. This difference in Western blot profiles may be useful in serologically differentiating *E. canis* and CGE infections, of particular importance, given that CGE has not been cultured in vitro and the antigen is not available for conducting homologous serologic tests. Although *Ehrlichia equi* can experimentally establish an infection in granulocytes of dogs (14), Western blot profiles of equine anti-*E. equi* serum against various *Ehrlichia* species (17) differed from those of canine anti-*E. canis* or anti-CGE serum. In a comparison of Western blot profiles of *E. risticii*, *E. sennetsu*, *E. canis*, *E. equi*, and *N. helminthoeca* antisera (17), CGE antisera were more closely related to those of *E. canis* than to those of *N.*

helminthoeca or to those of other *Ehrlichia* spp. These data agree with those of Anderson et al., who reported the closest genetic similarity of CGE to *E. canis* by comparison of the 16S rRNA gene sequence of the former with those of *E. canis*, *E. equi*, *Ehrlichia phagocytophila*, *E. sennetsu*, and *E. risticii* (1).

Sera collected from dogs naturally infected in Indonesia showed Western blot profiles almost identical to those of sera from dogs experimentally infected in the United States with an Oklahoma isolate of *E. canis*. As predicted from the frequent observation of clinical canine ehrlichiosis in Indonesia (19), our serologic study confirms that the disease on an Indonesian island was caused by *E. canis* or a closely related organism but not by CGE.

This study demonstrated that *E. canis* cultured in DH82 cells can induce a clinical disease similar to that induced by whole-blood transfusion or by tick attachment (3, 12). Tissue culturing was found more sensitive than direct observation of blood smears for detecting parasitemia. Since *E. canis* multiplies slowly, however, positive identification of parasitemia required 20 to 50 days of culturing. Prednisone treatment shortened the culturing period required for isolation, although the isolation was not consistent. Thus, to identify the carrier status of dogs infected with *E. canis*, prednisone treatment and repeated samplings several days apart are suggested.

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