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Serological Diagnosis of Tropical Canine Pancytopenia by Indirect Immunofluorescence

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An indirect fluorescent-antibody test for detection and titration of antibodies to *Ehrlichia canis*, the causative agent of tropical canine pancytopenia, has been described. The organism propagated by an in vitro technique in canine blood monocytes served as an antigen in the test. The specificity of the test was revealed by absence of cross-reactivity between the antigen and sera from dogs infected with various common pathogens and specific sera against eight rickettsial species. The accuracy of the test was ascertained by isolation of the organism from reactor dogs located in and outside the United States. Histopathological examination of nine reactor dogs revealed plasmacytosis of meninges and kidneys in eight of them.

Tropical canine pancytopenia (TCP) is a tick-borne rickettsial disease of dogs caused by *Ehrlichia canis* (7, 8). The organism was first recovered from dogs in Algeria in 1935 (2). Infections with *E. canis* are now known to occur in various parts of the world, including the United States (3, 6, 10). The acute form of the disease is characterized by pancytopenia, particularly thrombocytopenia, anorexia, emaciation, dehydration, and fever. Epistaxis may be a terminal manifestation of the disease. In acutely infected animals the organism can be detected in the cytoplasm of circulating monocytes as an inclusion body referred to as a morula. The dogs which recover remain carriers of the infection, but the organism usually cannot be demonstrated in films of peripheral blood. These animals may serve as a source of the infection for susceptible dogs in areas in which a suitable arthropod vector exists. The need for a serological test which can be used for detection of *E. canis* antibody was emphasized by various investigators. However, the lack of a method for production of ehrlichial antigen has hampered development of such a test (1, 3).

Recently Nyindo et al. (9) developed a method for propagation of *E. canis* in monocyte cell cultures and demonstrated an antibody to the organism in blood serum of infected dogs. The method provided a means whereby the antigen of this organism can be produced in quantities needed for development of serodiagnostic procedures for TCP.

This paper describes an indirect fluorescent-antibody (IFA) test for detection and titration of antibodies to *E. canis*. The test is specific and applicable to diagnosis of experimentally and naturally induced TCP.

MATERIALS AND METHODS

Preparation of antigen. The organism used in this study was recovered in 1969 from blood of a German Shepherd dog with signs of TCP in Florida (6). Infected monocyte cell cultures grown in 3-oz plastic flasks for 10 to 14 days according to the method of Nyindo et al. (9) were used as the source of antigen. Approximately 25 to 40% of the cells in these cultures were infected with *E. canis*. The fluid portion of the culture was removed and to each flask 2 ml of 0.02% ethylenediaminetetraacetate (tetrasodium salt) in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS), *p*H 7.2, was added. The flask was agitated on an electric rotator (Fisher Scientific Co.) at 170 oscillations per min at 37 C for 40 min. The contents were poured into a 5-ml conical glass tube and centrifuged at 206 × *g* for 5 min. The supernatant fluid was replaced with distilled water containing 1.75% bovine serum albumin (BSA) fraction V (Armour Pharmaceutical Co.), cells were gently brought into suspension, and the tube was centrifuged as above. The washing procedure was repeated, 0.25 ml of 1.75% BSA solution was added, and the final cell suspension (antigen) was prepared. The suspension was placed on the slide in six spots of 0.025-ml volume arranged

in two rows. The antigen spots were dried at 37 C for 1 hr and the slides were separated by porous paper, wrapped in aluminum foil, and stored at −65 C.

Fluorescein-conjugated anti-dog globulin. Antiserum was produced in two healthy rabbits inoculated with normal dog gamma globulin prepared by precipitation with ammonium sulfate in accordance with the method described by Goldman (4).

The gamma globulin was extracted from the immune rabbit serum by precipitation with 15% (w/v) sodium sulfate solution. The precipitate was dissolved in a volume of 0.15 M sodium chloride solution equal to 20% of the original serum volume and dialyzed overnight against 0.175 M sodium phosphate buffer (*p*H 6.3) at 3 C. The dialyzed protein was separated in diethylaminoethyl-cellulose equilibrated in 0.0175 M phosphate buffer (*p*H 6.3). The protein concentration was adjusted to 10 mg/1 ml and labeled with fluorescein isothiocyanate (0.033 mg/mg of protein) dissolved in 0.5 M carbonate buffer solution (*p*H 9.5). The mixture was stirred at 22 C for 2 hr and then passed through a Sephadex G-25 column (Pharmacia Fine Chemicals) to remove free fluorescein isothiocyanate. The conjugate was then absorbed for 30 min at 22 C with lyophilized bovine spleen powder in a ratio of 1 ml of conjugate to 10.0 mg of the powder. The conjugate was stored in 0.25-ml samples at −65 C.

Test sera. (i) Time sequence sera were collected from experimentally infected dogs beginning with the day of infection and during periods from 3 to 18 months thereafter. (ii) Single serum samples were collected from 61 U.S. military sentry dogs located in Southeast Asia where clinical TCP was diagnosed; from 6 military dogs at Lackland Air Force Base, Texas; from 21 dogs experimentally infected at Division of Veterinary Medicine, Walter Reed Army Institute of Research; from 13 experimentally infected and 16 noninfected dogs at the College of Veterinary Medicine, Univ. of Ill. Isolants from four diverse geographic regions (Puerto Rico, Virgin Islands, Florida, and Southeast Asia) were used to infect individual dogs from which the above sera were obtained. (iii) Sera were collected from dogs infected with *Leptospira canicola* (10 samples), *Brucella canis* (5 samples), canine herpesvirus (6 samples), parainfluenza virus SV 5 (15 samples), distemper virus and hepatitis virus (45 samples), and *Neorickettsia helminthoeca* (2 samples). (iv) Guinea pig sera were used which were specific to eight species of rickettsia, including: *Rickettsia tsutsugamushi* (Gilliam strain), *R. rickettsii*, *R. canada*, *R. burnettii*, *R. mooseri*, *R. akari*, psittacosis agent, and Tatlock agent, a rickettsia-like organism isolated from, and pathogenic for, the guinea pig (11). For use in the test, all sera were diluted 1:10 with PBS, *p*H 7.2. Serial twofold serum dilutions were prepared when titer was to be established.

Test procedure. Antigen slides were removed from the freezer and placed in a desiccator jar over calcium chloride. The jar was evacuated and slides were maintained in it for 1 hr at 22 C. The slides were then fixed in absolute acetone at 22 C for 5 min and allowed to dry for 15 min, and a circled area (1 cm in diameter) was flooded with test serum. Appropriate positive and negative control sera were included with each run. The slides were placed in a humidified chamber and incubated at 37 C for 30 min; they were rinsed twice for 5 min each time in PBS, rinsed for 5 min in distilled water, and air-dried, and then the fluorescein-conjugated anti-canine globulin was applied. Incubation and rinse procedures were repeated as above. Mounting fluid containing 9 parts of glycerin and 1 part of PBS was placed on each slide, and all six antigen spots were covered with a cover slip (22 by 40 mm). The slides were examined on a microscope equipped with an ultraviolet light source.

Inoculation of dogs and histopathological findings. A correlation between the presence or absence of indirect immunofluorescence reaction and actual infectivity status of the dog being examined was attempted in a few instances by (i) inoculating whole blood (5 to 20 ml) from test subjects into susceptible recipients and demonstrating TCP in the latter, and (ii) determination of specific microscopic tissue abnormalities in reactor dogs. The presence of plasmacytosis in the meninges and kidneys was considered to be a consistent manifestation of TCP.

RESULTS

The appearance of the fluorescence reaction in infected cells treated with serum from a dog with TCP is shown in Fig. 1a through d. The antigens detected in the cytoplasm of these cells were those of mixed populations of elementary and initial bodies (Fig. 1a), singly occurring morula (Fig. 1b), and multiple morulae (Fig. 1c). In each microscope field there were also cells containing apparently soluble antigen diffusely distributed throughout the cytoplasm (Fig. 1d). Fluorescence reaction of an entire microscope field viewed at lower magnification is shown in Fig. 2. No fluorescence of any kind was noted in or outside the cells when sera from *E. canis*-free dogs were used in the test.

The titer of sera collected from 10 experimentally infected dogs during periods from 3 to 18 months after exposure ranged from 1:10 to 1:640. The earliest reactions were demonstrated 11 to 28 days after inoculation. Three examples, indicating a relation between the antibody titer and an increase of body temperature are given in Fig. 3. In all three instances febrile reactions preceded the rise of antibody titer. Transitory febrile episodes were noted later in the infection. German Shepherd dog (GS-2) died with typical signs of TCP 90 days after exposure; Beagle dog (A-80) was killed when moribund approximately 1 year after infection, and Beagle dog (A-45) was a chronic carrier when testing was discontinued approximately 18 months after infection. At the time of death, GS-2 had an antibody titer of 1:80, whereas the titer of A-80 fell below detectable level.

The results of the test on three groups of dogs

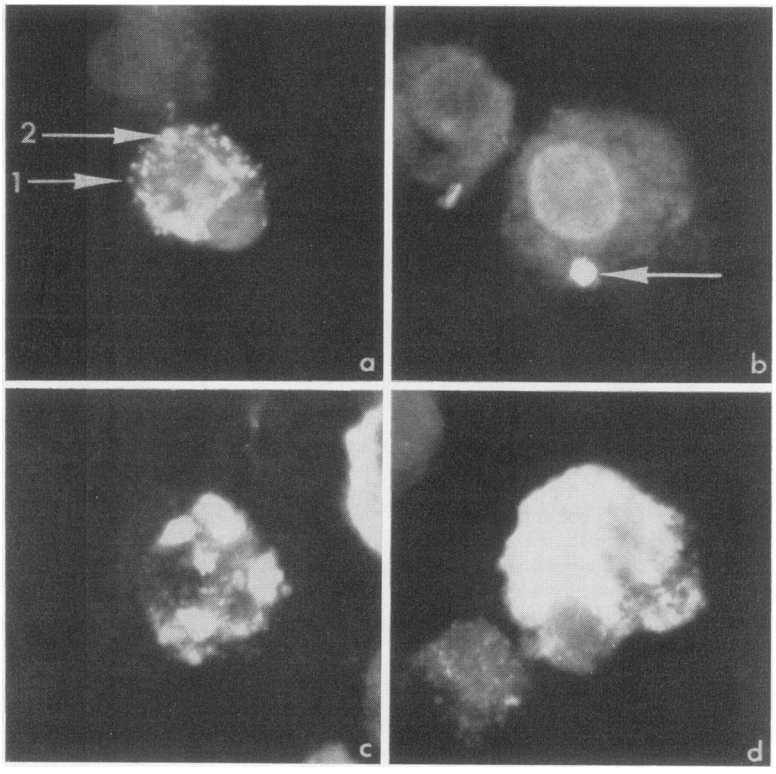

FIG. 1. *Developmental forms of the organism depicted in the indirect fluorescent-antibody test: mixed populations of elementary and initial bodies (a, arrows 1 and 2, respectively); a single morula (b, arrow); multiple morulae (c); and apparently soluble organismal antigens (d). ×540.*

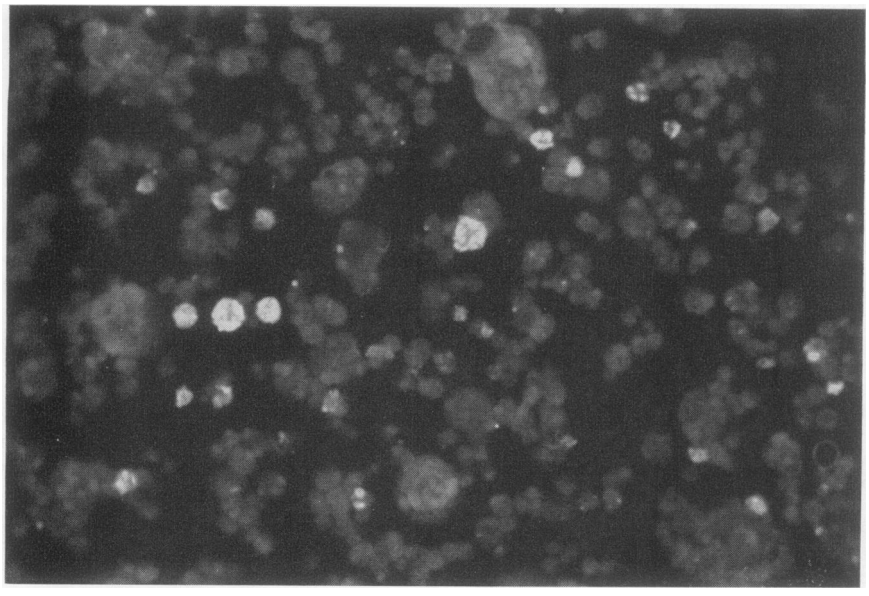

FIG. 2. *The appearance of a positive reaction observed in the indirect fluorescent-antibody test. Note fluorescence in cells infected with E. canis. ×250.*

using 1:10 serum dilutions are given in Table 1. In the experimentally induced disease group all 34 animals reacted in the test. The blood from 27 of these animals was inoculated into susceptible dogs. All recipients developed the infection. Of 61 dogs from regions of Southeast Asia, where naturally occurring disease was reported, 24 reacted in the test. The organism was recovered from two reactor dogs but not from two nonreactors. One reactor was detected among six dogs from Lackland Air Force Base, Texas. The dog which received the blood from this reactor developed TCP 10 days after inoculation. None of the 26 dogs from Georgia and Illinois, where the disease is not known to occur naturally, reacted in the test. Attempts to recover the organism from seven negative dogs failed.

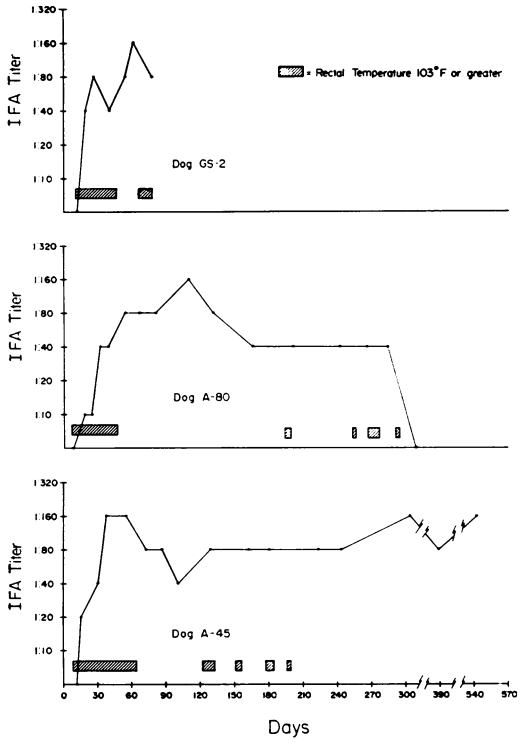

FIG. 3. *Relation between antibody titer detected by the indirect fluorescent-antibody test and the febrile response in three dogs experimentally infected with E. canis: GS-2, German Shepherd dog, died 90 days after exposure; A-80, Beagle dog, killed when moribund approximately 1 year after infection; A-45, Beagle dog, a chronic carrier at the time testing was discontinued approximately 18 months after infection.*

Eight of nine serologically positive dogs showed plasmacytosis of the meninges and kidneys at the time of their death (Fig. 4).

None of the specific sera against common dog pathogens and sera against eight rickettsial species reacted in the test (Table 2).

DISCUSSION

The results, in general, indicate that the IFA test is applicable to both experimental laboratory and field epidemiological investigation of TCP. In experimentally infected dogs the period prior to detection of antibodies varied from 11 to 28 days. The analysis of the inoculation data indicates that this variation is apparently due to individual animal responses rather than to the volume of inoculum used. From 10 experimentally infected animals, monthly blood subinoculations were made into susceptible dogs. The latter animals consistently developed clinical infections, demonstrating the existence of active carrier state in the donor animal. A further indication that the positive test may be indicative of an active infection was provided by transmission of the infection from three naturally occurring reactor dogs into susceptible dogs.

The test antigen (Florida isolant) detected anti-

TABLE 1. *Comparison of results of the indirect fluorescent-antibody (IFA) test and actual recovery of the organism*

TCP	Location	IFA		Recovery of organism			
				IFA^{+a}		IFA^{-a}	
		Tested	Positive	Attempted	Accomplished	Attempted	Accomplished
Experimentally induced	Walter Reed	21[b]	21	19	19	0	0
	U. of Ill.	13	13	8	8	0	0
Naturally occurring	Southeast Asia	61	24	2	2	2	0
	Texas	6	1	1	1	0	0
Not known to occur	Atlanta, Ga.	10	0	0	0	4	0
	Urbana, Ill.	16	0	0	0	3	0

[a] IFA^+ and IFA^-, Positive and negative indirect fluorescent-antibody test, respectively.
[b] Number of dog.

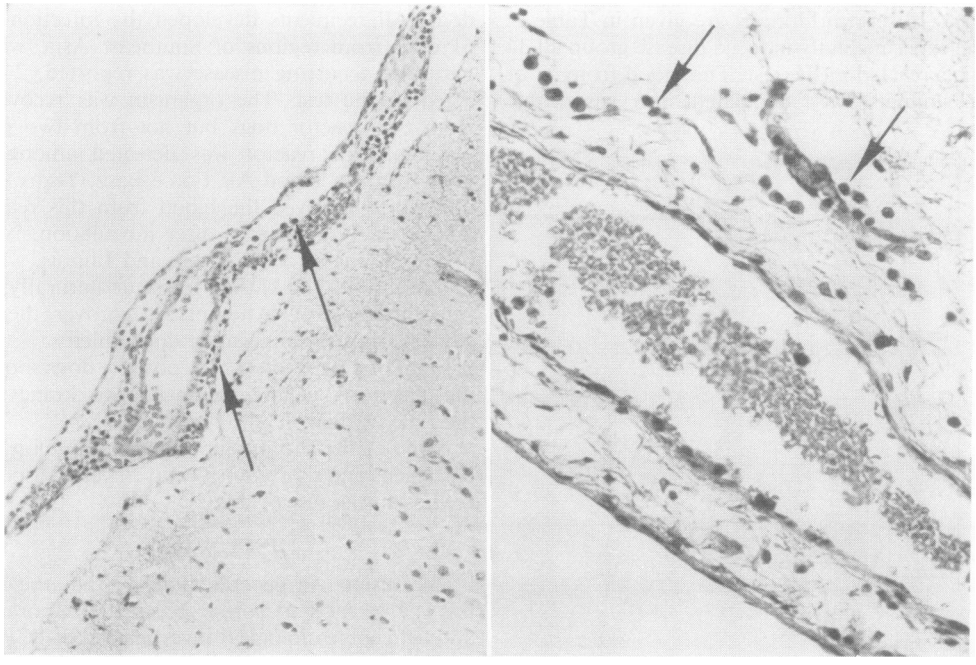

FIG. 4. *Mononuclear infiltrate in meninges of a dog affected with tropical canine pancytopenia. This infiltrate is particularly prominent about veins (left) ×250 and consists primarily of plasma cells (right) ×600 (hemolysin and eosin stain).*

TABLE 2. *Specificity of the indirect fluorescent-antibody (IFA) test for tropical canine pancytopenia (TCP)*

Antisera		No. of dogs	IFA for TCP (1:10 serum dilution)
Agent	Titer		
Leptospira canicola	>1:200 (Agglutination lysis)	10	Negative
Brucella canis	>1:400 (Tube agglutination)	4	Negative
Canine herpesvirus	>1:32 (Serum neutralization)	6	Negative
Parainfluenza virus SV_5	1:16 (Serum neutralization)	15	Negative
Neorickettsia helminthoeca	No test—animal known positive	2	Negative
Distemper virus and hepatitis virus	No test—immunized with live attenuated viruses	45	Negative
Rickettsia tsutsugamushi	>1:20,000 (IFA test)	1[a]	Negative
R. rickettsii	>1:20,000 (IFA test)	1[a]	Negative
R. canada	>1:20,000 (IFA test)	1[a]	Negative
R. burnettii	>1:20,000 (IFA test)	1[a]	Negative
R. mooseri	>1:20,000 (IFA test)	1[a]	Negative
R. akari	>1:20,000 (IFA test)	1[a]	Negative
Psittacosis agent	>1:20,000 (IFA test)	1[a]	Negative
Tatlock agent (rickettsia-like agent of guinea pig origin)	>1:20,000 (IFA test)	1[a]	Negative

[a] Guinea pigs were used instead of dogs.

bodies in dogs infected with isolants from Puerto Rico, Virgin Islands, and Southeast Asia, indicating serologic similarity among *E. canis* organisms from different geographic areas. In most dogs the antibody titer persisted during the entire observation period. However, in the terminal case, Beagle A-80 (Fig. 3), the titer sharply subsided below 1:10 during the last week. This could have been due to reduced immunological responsiveness apparently caused by a total exhaustion of the bone marrow as revealed by histopathological examination of terminal TCP cases (5). The role of

the immune response in the pathogenesis of TCP may be further investigated by careful correlative study of clinical-pathological manifestations and variations in IFA titer. Plasmacytosis of the meninges and kidneys appears to be a characteristic pathological manifestation associated with TCP.

A relatively high incidence of reactors found among the military dogs in Southeast Asia where the disease was diagnosed and serious losses occurred (6), indicates applicability of the test to epidemiologic investigation of TCP. Thus, the test may be used to determine the incidence of the disease in the U.S., particularly in the areas where the clinical cases have been described. Because of striking clinical resemblance between canine distemper and TCP, it would be of interest to examine by the IFA test suspected distemper cases, especially those occurring in tick-infested regions.

The test revealed no cross-serological relation between *E. canis* and any of the common canine pathogens. Also, none of the high-titer specific sera produced against eight rickettsial species reacted in the test. Recent electron microscopy studies by Hildebrandt et al. (*submitted for publication*) revealed that certain developmental forms of *E. canis* are similar but not identical with those of the agents belonging to psittacosis-lymphogranuloma venereum group of agents. Apparent lack of serological relationship between *E. canis* and the above-described rickettsiae provides further support that the agents of the genus *Ehrlichia* might be taxonomically a unique rickettsial group.

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