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CONTROL OF HEMOTROPIC DISEASES OF DOGS.(U)
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CONTROL OF HEMOTROPIC DISEASES OF DOGS
Annual Progress Report

January 1, 1976 - December 31, 1976

Supported by
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Washington, DC 20314

Contract No. DADA 17-70-C-0044

College of Veterinary Medicine
University of Illinois
Champaign-Urbana, Illinois 61801

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potential of the brown dog tick Rhipicephalus sanguineus as a reservoir of Ehrlichia canis was investigated. R. sanguineus adults harbored and efficiently transmitted E. canis to susceptible dogs for as long as 155 days after detachment as engorged nymphs from a dog experiencing acute ehrlichiosis. Two modifications of the original tissue culture technique for the propagation of E. canis were developed to study the effect of serum and macrophages from infected dogs on growth and development of E. canis and to provide continuous production of large quantities of E. canis antigen. A combination of chemotherapy and serologic monitoring of disease was found to be a useful method for field control of tropical canine pancytopenia.

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I. GENERAL INFORMATION

Severe losses and prolonged morbidity effects which tick-borne ehrlichiosis and babesiosis cause to the U.S. Army dogs prompted initiation of this project. Thus, initially the project was strictly problem oriented toward acquiring knowledge needed for the development of control measures for these diseases. Valuable research findings were made which provided for specific recognition of these diseases in the field and for monitoring the effects of chemotherapeutic and prophylactic measures. Implementation of control measures is being conducted in close collaboration with the Division of Veterinary Resources and the Division of Pathology, Walter Reed Army Institute of Research and its overseas Medical Research Units in Kuala Lumpur, Malaysia, and Bangkok, Thailand. During the past year, an additional request for collaborative assistance was received from the U.S. Air Force by way of the Walter Reed Army Institute of Research. As of November 17, 1976, 528 sera (representing more than 2,000 serologic tests) from dogs assigned to protect various U.S. Air Force bases in Southeast Asia and elsewhere were examined for evidence of exposure to ehrlichia.

As research on the 2 diseases progressed, it became apparent that the pathogenesis of canine ehrlichiosis, particularly the syndrome termed tropical canine pancytopenia (TCP), was immunopathologic in nature. Cell-mediated and humoral responses related to infection with Ehrlichia canis have been revealed, and their properties described. These findings may provide information on the occurrence and mechanism of similar immunopathologic manifestations associated with various infectious and non-infectious diseases of man and animals in which thrombocytopenia is a prominent pathologic manifestation.

II. SCOPE OF RESEARCH PRIOR TO 1976

A. Agents Used in the Study

Ehrlichia canis is a type species for a group of small pleomorphic rickettsias found predominantly in circulating monocytes of infected dogs. The organism is naturally transmitted by ticks and causes canine ehrlichiosis characterized by fever, anorexia, leukopenia, and thrombocytopenia. A syndrome typified by the occurrence of epistaxis in dogs infected with E. canis was termed tropical canine pancytopenia (TCP).¹ The etiologic agent was first recognized in tick-infested dogs in Algeria.² A peculiar strain of E. canis which predominantly invades circulating neutrophils and eosinophils³ rather than monocytes which are commonly affected has been isolated recently in the U.S. Both strains form inclusions (morulae) in host cells. This latter strain resembles, and may be identical with, Ehrlichia equi, the etiologic agent of equine ehrlichiosis. Infection of horses with E. equi is manifested by fever, anorexia, stiffness and edema of the limbs, and thrombocytopenia. The agent is found in the cytoplasm of neutrophils and eosinophils and is easily detectable in these cells at the time of acute infection. The taxonomic classification of E. equi has not been clearly defined, however, it has been

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1. Huxsoll, D. L. and Hildebrandt, P. K. Tropical Canine Pancytopenia (Ehrlichiosis). In: R. W. Kirk (Editor), Current Veterinary Therapy, V. Small Animal Pract. W. B. Saunders Co., Philadelphia PA pp. 369-371.
 2. Donatien, A. and Lestoquard, F. Existence in Algerie d'une Rickettsia du chien. Bull. Soc. Pathol. Exot., 28 (1935): 418-419.
 3. Ewing, S. A. and Philip, C. B. Ehrlichia-like Rickettsiosis in Dogs in Oklahoma and Its Relationship to Neorickettsia helminthoeca. Am. J. Vet. Res., 27 (1966): 67-69.

proposed that the organism should be grouped with the agents of the genus Ehrlichia, Order Rickettsiales.⁴ The ability of the agent to infect a variety of animal species, including dogs, distinguishes it from other previously described ehrlichial agents, i.e., Ehrlichia canis, characterized as having a narrow host range.⁵

Two strains of canine babesia were used for immunoserologic studies in collaboration with various U.S. Army and Air Force components.⁶ These are Babesia canis, a domestic and a European strain, and Babesia gibsoni, isolated by the U.S. Army Medical component SEATO Laboratory, Bangkok, Thailand.

B. Immunologic Studies

Development in our laboratory of a method for in vitro cultivation of Ehrlichia canis, the causative agent of TCP, provided a means for production of antigens needed in studies of the immune response in infected dogs.⁷ In an effort to achieve urgently needed diagnostic procedures for TCP, the antigen generated in cell culture was successfully applied to development of an indirect fluorescent antibody (IFA)

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4. Stannard, A. A., Gribble, D. H. and Smith, R. S. Equine Ehrlichiosis: A Disease with Similarities to Tick-borne Fever and Bovine Petechial Fever. Vet. Rec., 84 (1969): 149-150.
 5. Lewis, G. E. Jr., Huxsoll, D. L., Ristic, M. and Johnson, A. J. Experimentally Induced Infection of Dogs, Cats, and Nonhuman Primates with Ehrlichia equi, Etiologic Agent of Equine Ehrlichiosis. Am. J. Vet. Res., 36 (1975): 85-88.
 6. Ristic, M., Lykins, J. D., Smith, A. R., Huxsoll, D. L. and Groves, M. G. Babesia canis and Babesia gibsoni: Soluble and Corpuscular Antigens Isolated from Blood of Dogs. Exp. Parasit., 30 (1971): 385-392.
 7. Nyindo, M. B. A., Ristic, M., Huxsoll, D. L. and Smith, A. R. Tropical Canine Pancytopenia: In vitro Cultivation of the Causative Agent - Ehrlichia canis. Am. J. Vet. Res., 32 (1971): 1651-1658.

test for diagnosis of the disease.⁸ The test provided an accurate and sensitive means for laboratory and field studies of TCP. By use of this diagnostic method, the disease was detected in many parts of the U.S., South America, and Southeast Asia. Three major practical applications of the IFA test have been: (1) the isolation and identification of E. canis from various geographic regions; (2) detection of TCP-free dogs for safe return to the U.S. from Southeast Asia, and (3) monitoring the effect of chemotherapeutic procedures being tested as mass control measures.

A similar test was developed for diagnosis of equine ehrlichiosis caused by Ehrlichia equi.⁹ The antigen used in the latter test was isolated from equine buffy coat leukocytes collected during the acute phase of the experimentally-induced disease. Serologic cross-reaction between E. canis and E. equi was established using the IFA method.

Antigens of the 2 organisms were used in the detection and measurement of cell-mediated immunity (CMI) in dogs and horses infected with E. canis and E. equi, respectively. Twelve German shepherd dogs and 5 beagles were experimentally infected with E. canis and their cell-mediated and humoral responses studied over a 120-day period. Fifty percent of the German shepherd dogs and 80% of the beagles were designated as positive in the leukocyte migration inhibition (LMI) test using peripheral blood. All dogs developed high titers

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8. Ristic, M., Huxsoll, D. L., Weisiger, R. M., Hildebrandt, P. K. and Nyindo, M. B. A. Serological Diagnosis of Tropical Canine Pancytopenia by Indirect Immunofluorescence. *Infect. Immun.*, 6 (1972): 226-231.
 9. Nyindo, M. B. A. Immune Responses to Ehrlichia canis and Ehrlichia equi in Experimentally Infected Dogs and Ponies. PhD thesis, University of Illinois, Champaign-Urbana, IL (1975).

in the IFA test. Treatment of infected dogs with anti-canine lymphocyte serum (ALS) did not alter the course of infection in beagle or German shepherd dogs. Data derived from the use of 2,4-Dinitrochlorobenzene (DNCB) and Old Tuberculin (OT) did not produce conclusive evidence that infection with E. canis alters the preinfection level of a state of immunocompetence.⁹

C. Immunopathologic Studies

The pathogenesis of thrombocytopenia, a consistent pathologic manifestation of infections caused by E. canis, has been studied but the mechanism has not been clearly defined. On the basis of the finding of hypergammaglobulinemia and plasmacytosis, an immunopathologic mechanism of thrombocytopenia in TCP was proposed.¹⁰ In a recently completed study, lymphocytes from dogs infected with E. canis were shown to exert a cytotoxic effect on autologous monocytes using ⁵¹Cr labeled target cells.¹¹ The 7 dogs were tested for autologous cell-mediated monocytoxicity prior to inoculation and a pre-inoculation base line of 0 to 5.5% was established. Between day 10 and 15 post inoculation (PI), cytotoxicity reached a peak (79%) and gradually declined, showing 12 to 34% values at 60 days PI. A direct relationship between cell-mediated monocytoxicity and thrombocyte count was demonstrated; the greatest degree of cytotoxicity coincided with low platelet counts during the acute phase of disease.

10. Buhles, W. C. Jr., Huxsoll, D. L. and Ristic, M. Tropical Canine Pancytopenia: Clinical, Hematologic, and Serologic Response of Dogs to Ehrlichia canis Infection, Tetracycline Therapy, and Challenge Inoculation. The J. of Infect. Dis., 130 (1974): 357-367.
11. Kakoma, I., Carson, C. A., Ristic, M., Huxsoll, D. L., Stephenson, E. H. and Nyindo, M. B. A. Autologous Lymphocyte Mediated Monocytoxicity in Tropical Canine Pancytopenia. In press, Am. J. Vet. Res. (1976).

Maximum cytotoxicity preceded the lowest platelet counts by 1 to 2 days. By 45 days PI, designated as the onset of the chronic phase of disease, platelet counts began to increase while monocytoxicity fluctuated at a low level. Monocytoxicity declined during the chronic phase, but persisted at a significant level of 35%. The effect of various dilutions of immune serum on cytotoxicity was studied. Addition of immune serum resulted in minimal enhancement of monocytoxicity. The cytotoxic effect was directly dependent on the concentration of effector cells, being optimal at an effector cell-target cell ratio of 100:1. The degree of cytotoxicity was not significantly affected by treatment of the lymphocytes with anti-canine immunoglobulin and complement.

III. REPORT OF RESEARCH COMPLETED DURING THE YEAR 1976

A. A Platelet Migration Inhibition Test for Canine Ehrlichiosis:

Development of the test and its relationship to indirect fluorescent antibody test.

1. Introduction

Severe thrombocytopenia is a major part of the pancytopenia in canine ehrlichiosis. Platelet survival time is shortened presumably due to excessive platelet destruction.¹² The disease induces both cellular⁹ and humoral⁸ responses and it has been proposed that the immune response may cause pathologic changes.¹⁰

Recently a platelet migration inhibition (PMI) test¹³ was developed to measure antiplatelet antibody in human idiopathic thrombocytopenia. The present study was devised to investigate the applicability of this technique to the detection of antiplatelet activity in canine ehrlichiosis and to compare the kinetics of this response to that of the specific antibody detectable by the IFA test.

2. Materials and Methods

Collection of serum samples: Serum samples were collected from 9 adult beagle dogs known to be free from ehrlichiosis for use in the determination of the base line for the PMI activity. Four of the dogs were each inoculated with 10 ml of whole blood from a dog chronically infected with *E. canis*. Two dogs were also inoculated with 10 ml of normal dog blood to serve as

12. Smith, R. D., Ristic, M., Huxsoll, D. L. and Baylor, R. A. Platelet Kinetics in Canine Ehrlichiosis: Evidence for Increased Platelet Destruction as the Cause of Thrombocytopenia. *Infect. Immun.*, 11 (1975): 1216-1221.
13. Duquesnoy, R. J., Lorentzen, D. F. and Aster, R. H. Platelet Migration Inhibition: A New Method for Detection of Platelet Antibodies. *Blood*, 46 (1975): 741-747.

controls of possible anti-blood group and anti-histocompatibility response. Following inoculation, platelet levels were monitored and serum was separated, divided into 0.5 ml aliquots, inactivated at 56 C for 30 minutes and stored at - 65 C until used. Serum samples from dogs naturally infected with E. canis were also tested.

Preparation of platelets: A modification of the procedure described by Duquesnoy¹³ was used. Twenty ml of blood was drawn in 2 ml of 20% sodium citrate solution. The blood was centrifuged at 400 to 500 g for 10 minutes in siliconized polycarbonate tubes at room temperature. The upper three-fourths of the platelet-rich (PRP) layer was removed and centrifuged at 1000 g for 10 minutes. The platelet pellet was resuspended in 0.1 ml of supernate designated platelet poor plasma (PPP). The remaining PPP was inactivated at 56 C for 30 minutes and clarified by centrifugation at 7000 g for 30 minutes. In all the experiments, one dog was used as a standard source of platelets. Platelets collected from this donor dog did not react with preinfection serums of dogs used in the experiments.

Platelet migration inhibition test. Medium¹³ was prepared by mixing 3 parts of Eagle's minimum essential medium (MEM)^a with 2 parts of autologous PPP and 200 units of penicillin-streptomycin.^b Two-tenths of 1 ml of inactivated test serum was added to 0.2 ml of a suspension of platelets containing 1 to 2 x 10⁸ platelets.

^aGibco, Grand Island, NY.

^bInternational Scientific Industries, Cary, IL.

per ml. The mixture was incubated at room temperature for 1 hour. Microcapillary tubes (25 μ) were filled with platelet-serum mixture and heat sealed. After centrifugation at 1,000 g for 3 minutes, the capillary tubes were cut just above the interface between packed platelets and supernatant solution. The closed end of the stumps was embedded into silicone-grease in the center of a 25 mm cover slip which served as the floor of the migration chamber.^c The migration chambers were closed with a second cover slip adhered by silicone-grease. The space between the 2 cover slips was filled with the medium and the entry portals sealed with hot paraffin wax. All tests were carried out in triplicate and each chamber contained at least 3 stumps. The chambers were incubated at 37 C for 16 hours. The areas of migration was measured using a light microscope with a calibrated ocular micrometer at 4X magnification. The mean percent of migration inhibition relative to the area migrated by platelets incubated with normal dog serum in autologous serum was calculated for each test serum.

Indirect fluorescent antibody (IFA) test was carried out as described earlier.⁸

Scanning electron microscopy (SEM) on normal and inhibited platelets. Normal dog platelets were incubated with inhibitory serum for 1 hour at room temperature. A control portion of the same preparation of platelets was similarly incubated with normal

^cPhysical plant, University of Illinois, Urbana, IL.

dog serum. A small drop of preparation was placed on a cover glass and mixed with a drop of phosphate buffered saline (PBS). The cover slip was then fixed with 4% gluteraldehyde and washed with PBS for at least 15 minutes. The adherent cells were then dehydrated by successively covering them with 10, 25, 50, 75, 85, 95% ethyl alcohol and finally dehydrated twice in absolute alcohol. Each dehydration stage took 5 to 10 minutes. Cells were critically dried in a Samdri PVT-d^d apparatus (1 to 2 x 10⁵ torr) directly from the 100% ethanol. The cover glasses with dried cells were attached to stubs with the cells uppermost and given a coating of approximately 300 Å gold in a Denton DV-503 vacuum evaporator^d and viewed in a Hitachi - S 500^e scanning electron microscope at 20 KV and 20° tilt.

3. Results

The typical migration inhibition pattern is shown in Fig. 1 (a and b) comparing cells exposed to normal preinfection serum and to post infection serum obtained from the same dog in the acute phase of ehrlichiosis. In some cases inhibition as high as 99.8% was obtained.

In Fig. 2 (a and b), the appearance of normal and inhibited platelets revealed by scanning electron microscopy are shown. Uninhibited plates show numerous pseudopod formation whereas inhibited platelets are generally rounded and smooth and occasionally

^dDenton Vacuum Inc., Cherry Hill Industrial Center, Cherry Hill, NY.

^eHitachi Co., Japan.

show evidence of membrane damage and apparent shrinkage and loss of intracellular contents.

In Table 1 (a) the kinetics of the platelet migration inhibition (PMI) factor is compared with the development of the specific anti-E. canis antibody demonstrated by the IFA test in German shepherd dogs infected by inoculation of carrier blood. At 21 days after infection, dogs GS 20 and GS 19 were negative in the IFA test yet showed PMI activities of 94% and 98.8%, respectively. Thereafter activities in both the IFA and PMI tests were in evidence. Table 1 (b) shows the relationship of the IFA to the PMI test performed on sera from dogs naturally infected with E. canis. These dogs represent ehrlichiosis cases identified in and outside the U.S. In most of the samples tested, the PMI was positive when the IFA was positive. There was no direct relationship as to the titer of the tests. Serum samples (81-11, 78-8) had high PMI values but IFA titers of 1:320 whereas some samples with high IFA titers (1:1280) (#51-8, 78-5, 72-10) had low PMI values. None of the dogs inoculated with normal dog blood showed PMI or IFA response beyond the established base line of 0 to 15% for PMI test and 1:10 serum dilution for the IFA test. Finally, randomly selected E. canis-free dogs were also tested and shown to be negative in both tests (Table 1 (c)).

Fig. 3 shows the relationship between thrombocyte count and percent PMI activity in beagle dogs. Platelet counts began to decline in both dogs when high migration inhibition activity was demonstrable. In Fig. 4 the kinetic relationship between the IFA and PMI tests in a beagle dog is shown. By day 10 a PMI

of 34.5% was measured but the IFA titer was only 1:10. Peak migration inhibition of 45.5 was measured on day 45. The highest IFA titer of 1:1280 was attained at approximately the same time. Thereafter, the 2 activities began to fluctuate but had not returned to base line values by day 70 (the last day of the experiment).

Fig. 5 shows a general relationship between platelet level, IFA titer and PMI percent in one of the experimentally infected beagle dogs. An early activity of the PMI is evident. Both IFA and PMI attained maximum levels between 40 to 45 days post infection.

B. Preliminary Characterization of Platelet Migration Inhibition Factor(s).

Gel Filtration on Sephadex G-200. Serum from a dog positive in the PMI test was dialyzed against 0.15 M sodium chloride for 24 hours. Two ml of the dialyzed sample was applied on a 2.5 x 80 cm G-200 column equilibrated with 0.1 M Tris-HCl buffer containing 0.15 M sodium chloride. The column was eluted with the same buffer at the rate of 16 ml per hour. Optical density was monitored at 280 nM. Distinct peaks (I, II, III) rich in IgM, IgG, and albumin, respectively, were demonstrated and corresponding fractions were pooled (Fig. 6).

Results of the PMI Test on G-200 Fractions of Normal and Immune Sera.

The peaks were concentrated by pervaporation and reconstituted in normal dog serum. The reconstituted peaks were tested in the PMI test. The greatest degree of PMI (67%) was observed in the IgG peak followed by 31% of the IgM peak. Only 10.5% inhibition was observed

in the albumin-rich peak. Parallel studies using fractions of normal dog serum gave 3 to 11.5% inhibition in the IgG-rich peak but the IgM and albumin peaks were negative.

1. Discussion

The study described under A and B has demonstrated that dogs infected with Ehrlichia canis synthesize humoral factor(s) directed against normal canine platelets and detectable by a PMI test. The activity coincided with the severe phase of thrombocytopenia but remained at high levels even during the chronic phase of the disease. The antibody measurable by IFA does not seem to be directly associated with protection¹⁴ and may contribute to the pathogenesis of the disease since it relates to maintenance of the long-term carrier stage.¹⁴ The present study shows that PMI precedes the IFA response and may perhaps be one of the earliest events in platelet injury. An early appearance of PMI activity in IFA-negative samples from known infected dogs indicates that synthesis of specific antibodies and/or factors reactive in these tests proceeds at different time sequences. Preliminary unpublished studies have shown that normal canine platelets can totally absorb the factor(s) responsible for PMI activity without affecting the IFA titer. Thus the 2 activities apparently possess different immunologic specificities. Both IFA and PMI activity

14. Weisiger, R. M., Ristic, M. and Huxsoll, D. L. Kinetics of Antibody Response to Ehrlichia canis Assayed by the Indirect Fluorescent Antibody Method. *Am. J. Vet. Res.* 36 (1975): 689-694.

arose in response to infection with E. canis since dogs inoculated with blood from ehrlichia-free dogs failed to induce either response.

Platelet migration is a genuine physiologic phenomenon distinct from simple diffusion and Brownian movement¹⁵ and provides a good index of platelet function. Inhibition of platelet migration in vivo could have important pathogenic effects. An excessive sequestration of platelets in the vasculature and premature destruction by the reticuloendothelial system are among the various elements contributing to thrombocytopenia.¹⁶ Migration inhibition would interfere with the exit of the platelets from sites of synthesis and would also facilitate adherence of platelets to the endothelium in a manner analogous to the thrombocytopenia associated with Rocky Mountain spotted fever.¹⁷ Results of the SEM showed that the anti-platelet factor induces prominent surface changes on platelets and thus may render them more vulnerable to direct destruction by the reticuloendothelial system.

Although the in vivo applications of the platelet migration assay are speculative, the present findings strongly suggest that the pathogenesis of canine ehrlichiosis has an immunologic component in which platelets may be damaged directly.

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15. Lowenhaupt, R. W., Miller, M. A. and Glueck, H. I. Platelet Migration and Chemotaxis Demonstrated in vitro. *Thromb. Res.* 3 (1973): 477-480.
 16. Wilkins, R. J., Hurwitz, A. I. and Dodds-Laffin, W. J. Immunologically Mediated Thrombocytopenia in the Dog. *JAVMA*, 163 (1973): 879-882.
 17. Hand, W. L., Miller, J. B., Reinartz, J. A. and Stamford, J. P. Rocky Mountain Spotted Fever. *Arch. Intern. Med.* 125 (1970): 879-882.

The evidence of humoral and cell-mediated immune responses directed to platelets and monocytes in dogs infected with E. canis may explain the mechanism of the thrombocytopenia and leukopenia which are the most profound hematologic manifestations associated with pathogenesis of canine ehrlichiosis. The finding may relate to the pathogenesis and mechanism of diseases of man such as idiopathic thrombocytopenic purpura and thrombocytopenias accompanying various protozoan and rickettsial diseases, i.e., malaria, trypanosomiasis, babesiasis, dengue hemorrhagic fever, Rock Mountain spotted fever. Canine ehrlichiosis may prove to be a suitable experimental model for exploration of infectious and noninfectious thrombocytopenias occurring in man and animals.



Fig. 1 (a). Typical pattern of migration of uninhibited platelets when incubated with preinfection serum.



Fig. 1 (b). Migration pattern of platelets inhibited by serum from a dog with acute ehrlichiosis.



Fig. 2 (a). Scanning electron-micrograph of canine platelets incubated in preinfection serum. Note the typical pseudopod formation.



Fig. 2 (b). Scanning electron-micrograph of canine platelets incubated in the serum of a dog with acute ehrlichiosis.

Table 1 (a). Anti-platelet effect in serum of German shepherd dogs with experimental ehrlichiosis and its relationship with specific antibody (needle inoculation).

Days Post-Infection	Dog # GS 20		Dog # GS 19	
	IFA Titer	PMI %	IFA	PMI %
0	N	0	N	0
14	N	22	N	0
21	N	94	N	98.8
23	1:80	99.8	1:40	32.5
35	1:160	99.8	1:40	98.6
42	1:320	72	1:160	95.6
51	1:160	NT	1:160	54.0
69	1:320	47.6	1:80	NT
90	1:160	67	1:80	NT

Table 1 (b). Relationship between anti-platelet effect and IFA in serum from naturally infected dogs.

Dog #	IFA Titer	PMI %
51	1:1280	29.4
51-15	1:1280	80.0
78-8	1:320	85.8
78-5	1:1280	31.5
51-3	1:1280	48.3
81-11	1:320	52.6
72-10	1:1280	18.1
78-6	1:1280	63.2

Table 1 (c). Anti-platelet effect and IFA titer in randomly selected Ehrlichia-free dogs.

Dog #	IFA Titer*	PMI %**
19	N	5
21	N	3
A 52	N	8
67	N	2

N = negative

NT = not tested.

*First serial serum dilution used in the test was 1:10.

**Undiluted serum is used in the PMI test.

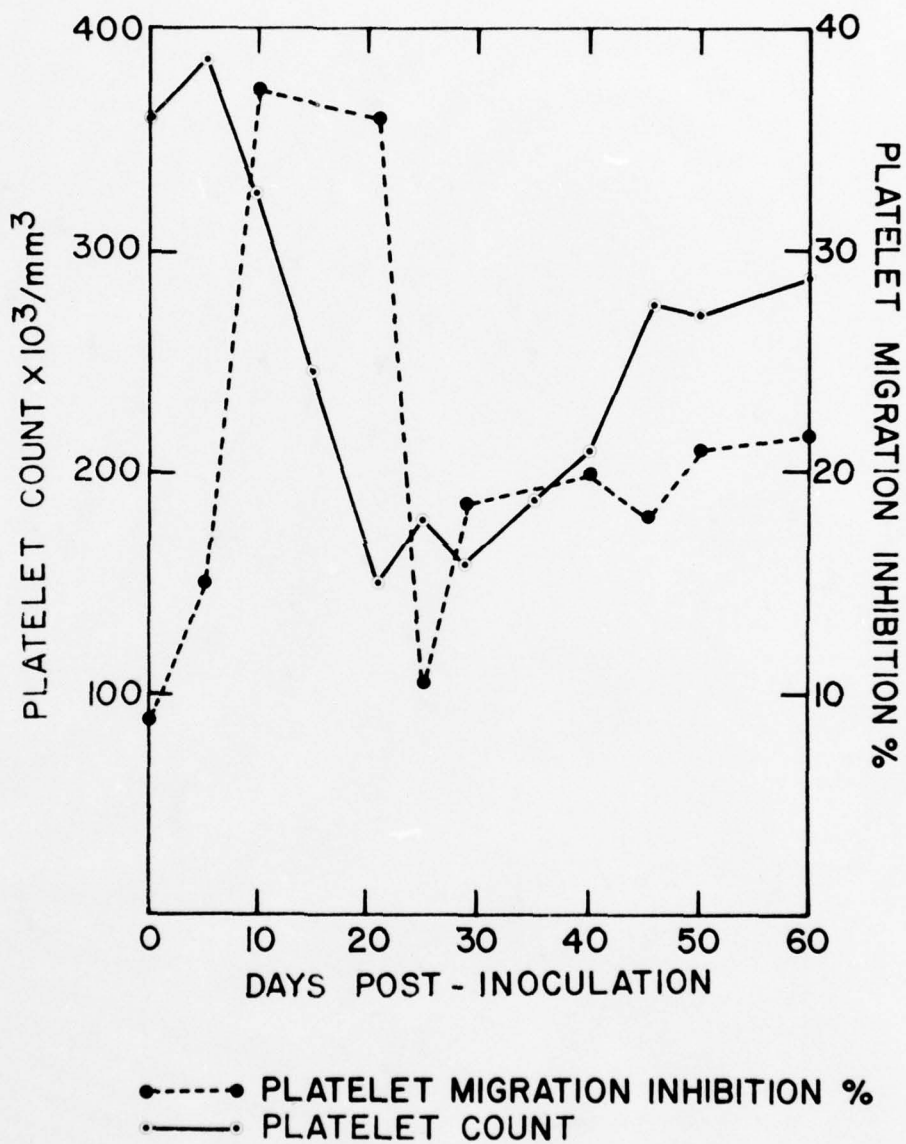


Fig. 3. Temporal relationship between platelet level and percent platelet migration inhibition in an *E. canis*-infected Beagle dog.

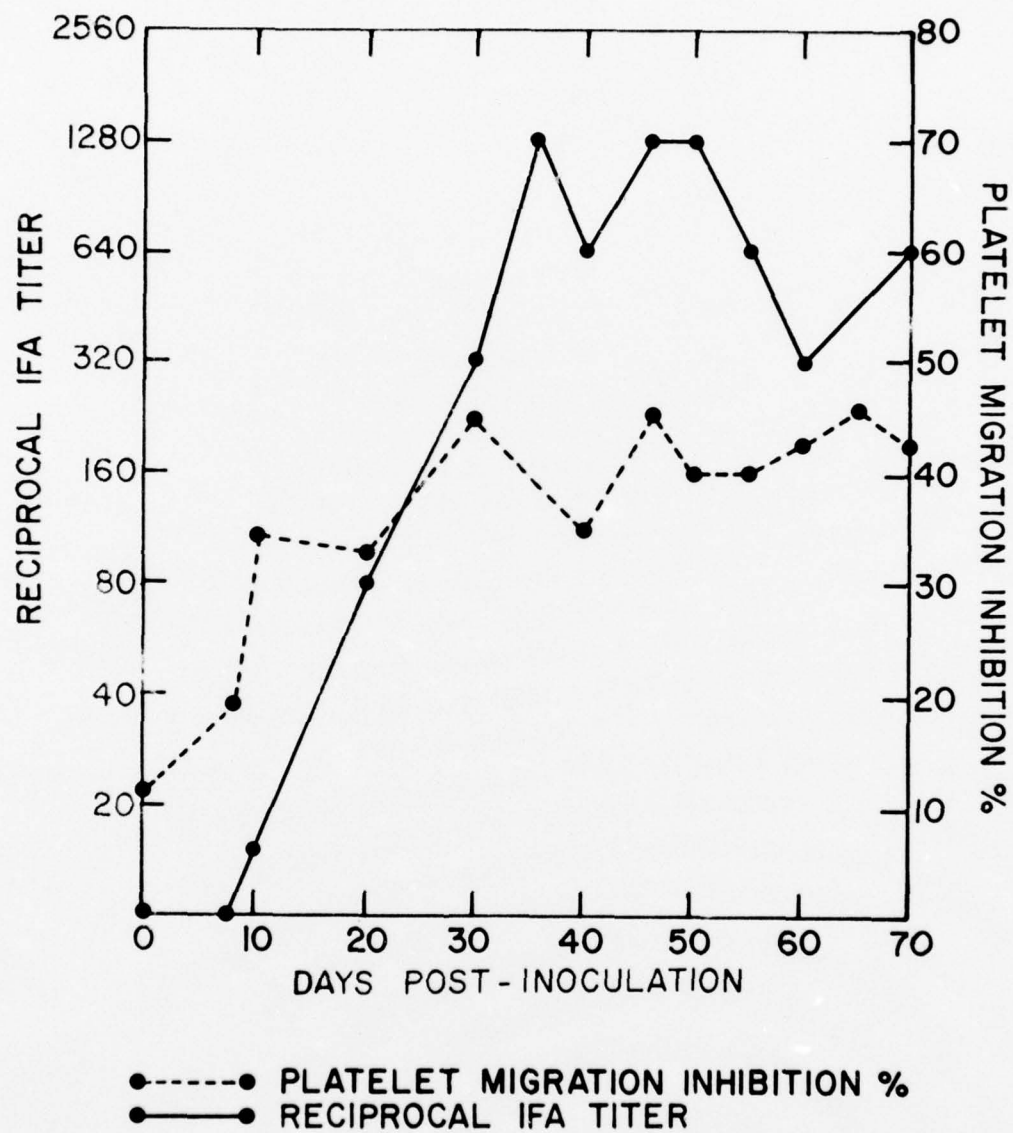


Fig. 4. The kinetic relationship between the platelet migration inhibition percent and the indirect fluorescent antibody titer in a Beagle dog infected with *E. canis*.

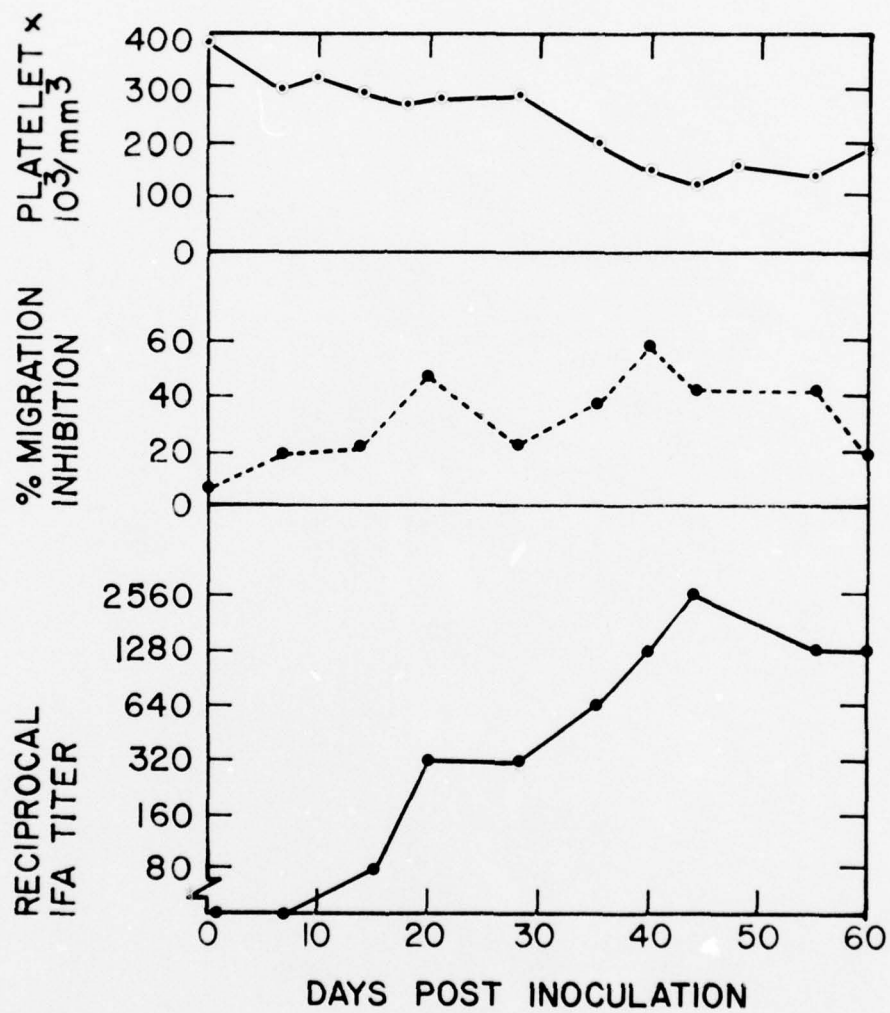


Fig. 5. An overall relationship between platelet levels, platelet migration inhibition percent, and indirect fluorescent antibody titer in a Beagle dog infected with *E. canis*.

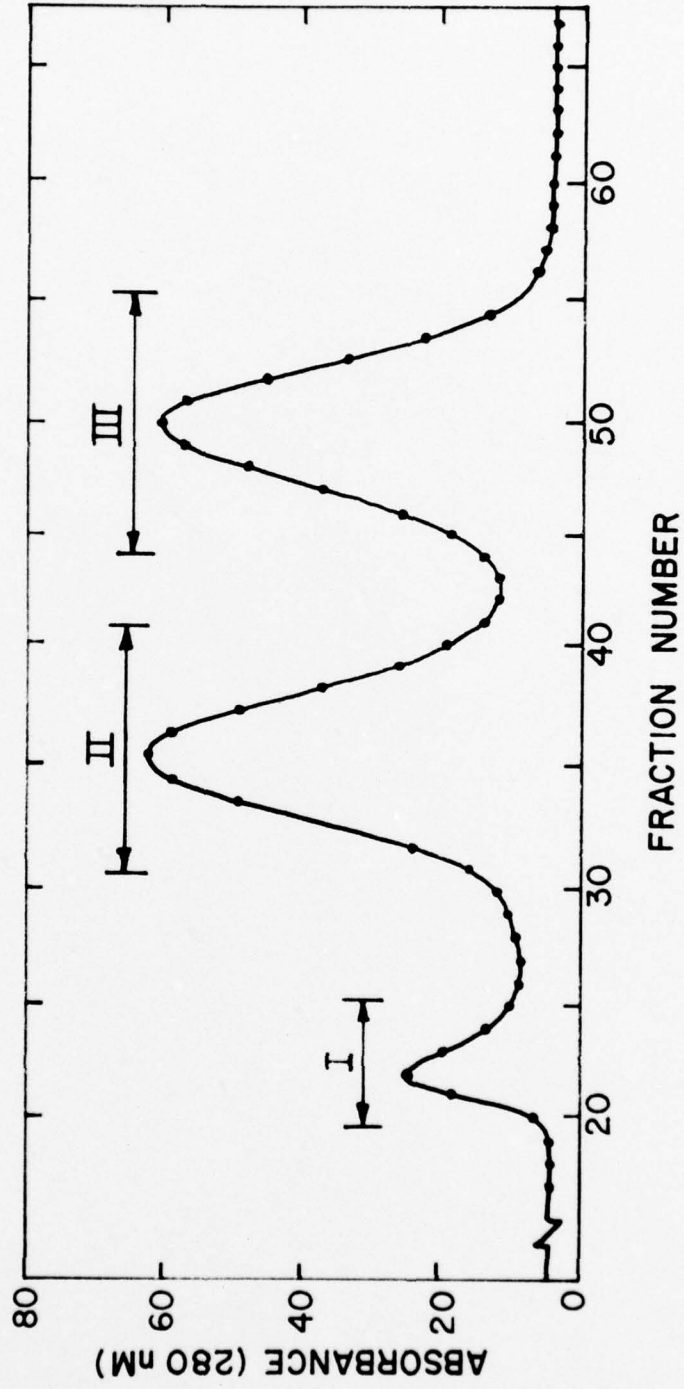


Fig. 6. The elution profile of whole serum from an *E. canis*-infected Beagle dog using Sephadex G-200 column. Preponderance of migration inhibition activity was found in peak II.

C. Isologous and Autologous Lymphocyte Mediated Anti-Platelet Cytotoxicity.

1. Introduction

It has been suggested¹² that during canine ehrlichiosis the platelet may be a target cell of the immune response. In human idiopathic thrombocytopenia, a disease with immunopathologic manifestations similar to those of canine ehrlichiosis, cellular immunity to autologous platelets was demonstrated (Wybran and Fudenberg, cited in Ref. 11). During canine ehrlichiosis, auto-reactive lymphocytes directed to monocytes are generated¹¹ and it was suggested that similar auto-reactivity may injure platelets.

The present study investigated the presence of auto-reactive and iso-reactive effector mechanisms directed against platelets in canine ehrlichiosis.

2. Materials and Methods

Animals. Ehrlichia canis-free beagle dogs between the ages of 9 months and 1 year were used. Four dogs were injected by intravenous inoculation of 10 ml of blood from an E. canis-carrier dog. A dog whose platelets were shown not to react with pre-infection lymphocytes or serum from dogs used in the experiment was chosen as the platelet donor throughout the experiment.

Preparation and labeling of platelets (target cells). Platelets were prepared by differential centrifugation of citrated whole canine blood as described in Section A. The platelet pellet was resuspended in 0.1 ml of platelet poor plasma (PPP) and labeled with ⁵¹Cr. A platelet pellet obtained from 20 ml of blood was first resuspended in 3 ml

of PPP and the platelets counted in a hemocytometer. The platelets were resuspended in 0.1 ml of PPP. Fifteen ml of a Ringer citrate-dextrose solution (7 parts Ringer's solution, 2 parts of a 3.12% trisodium citrate dihydrate, 1 part of a 5% dextrose) were added to the platelet suspension. After 10 minutes at room temperature the platelets were pelleted again at 1000 g for 5 minutes. Approximately 100 to 200 μ Ci of ^{51}Cr in isotonic saline was added. The mixture was incubated at room temperature for 30 minutes. The excess label was washed off with Ringer-citrate-dextrose by centrifugation at 1000 g for 5 minutes. After 3 washes, the platelet pellet was resuspended in PPP.

Preparation of lymphocytes (effector cells). Autologous lymphocytes were prepared as previously described¹¹ using a ficoll-hypaque gradient. Briefly, whole blood anticoagulated with 10% EDTA was diluted 1:2 in Hank's balanced salt solution (HBSS) medium and 22 ml layered on a 15 ml of ficoll-hypaque. After centrifugation at 1000 g for 1 hour at 4 C, the leukocyte layer was removed and resuspended in 20 ml of RPMI 1640 medium. The cells were then washed 3 times in RPMI 1640, counted, and their viability assessed by the trypan blue dye exclusion method. Generally 95% of cells were viable.

Preparation of serum. Whole blood was allowed to coagulate at room temperature for 1 to 2 hours and transferred to 4 C for at least 30 minutes. Serum was separated by centrifugation at 400 to 500 g at room temperature. Serum was collected weekly throughout

the experiment. The serum was inactivated at 56 C for 1/2 hour and then clarified at 7000 g for 20 minutes.

The indirect fluorescent antibody (IFA) test was performed as described earlier.⁸

The cytotoxicity test. Preliminary studies established a target cell:effector cell ratio of 1:2 as being optimal. Labeled platelets were distributed into siliconized tubes and effector cells and serum added. A group of tubes received lymphocytes alone while another group of tubes received lymphocytes and serum. A third group of tubes received target cells without any effector system. The final volume was adjusted to 1 ml with RPMI 1640 medium. The mixture was incubated at 37 C for 6 hours at the end of which period the tubes were centrifuged at 1000 g for 10 minutes. The supernatant fluid was assayed for radioactivity. The radioactivity in the supernatant fluid from target cells without the effector system represented spontaneous release. The pellet from these target cells was lysed by freezing and thawing 3 times. The radioactivity in this pellet was designated total release. All tests were set up in triplicate.

Percent specific ⁵¹Cr release was calculated from the formula:

$$\text{Specific } ^{51}\text{Cr release} = \frac{^{51}\text{Cr release from test} - \text{spontaneous release}}{100\% \text{ release} - \text{spontaneous release}} \times 100$$

3. Results

Table 1 shows the relationship between platelet count and anti-platelet cytotoxicity in isologous and autologous systems. The platelet count began to decrease 15 days post infection and started to recover by the 29th day. The lowest platelet level was reached on day 22 post infection. Isologous platelet cytotoxicity induced by lymphocytes only was first observed 5 days post infection, and

was highest on day 29. When immune serum was incorporated in the effector system, cytotoxicity was also demonstrable on day 5. Between day 29 and day 50 post infection, the presence of serum appeared to enhance the degree of cytotoxicity mediated by lymphocytes.

No valuable data were obtained from the autologous system since platelet yield was too low and the cells tended to clump during the acute phase of the disease.

Table 2 shows the relationship between isologous platelet cytotoxicity and the IFA titers. Reactions in the 2 tests appeared at approximately the same time post infection (day 5). Platelet cytotoxicity reached its peak by day 29, whereas the highest IFA titer recorded was on day 43 post infection.

4. Discussion

The role of direct immunologic injury to isologous platelets was demonstrated. Platelet damage by lymphocytes in the absence of serum predominated in the acute phase of disease and preceded the development of thrombocytopenia. Thus, early lymphocyte activation against platelet antigens may contribute to the pathogenesis of thrombocytopenia in canine ehrlichiosis via an antibody-independent pathway similar to the one proposed for monocytoxicity.¹¹ The role of antibody dependent damage to platelets was unclear since most of the activity was demonstrated during the recovery phase of the disease and persisted throughout the period of study. There was no clear relationship between the IFA titer and the severity of the disease. The antibody demonstrated by the IFA coincided

with the platelet cytotoxicity in contrast to the platelet migration inhibition activity which preceded the IFA response in most cases (Section A).

Table 1. Relationship between platelet count, and platelet cytotoxicity,^a isologous and autologous systems (dog # 510).

DAYS PI	PLT x 10 ³ /mm ³	Isologous System		Autologous System
		Lymphocytes Only	Lymphocytes and Serum	Lymphocytes and Serum
0	360	12.9%	5.6%	0
5	375	25.2%	10.2%	2
15	325	32.0%	16.4%	-*
22	150	30.0%	20.8%	13
29	210	36.0%	45.6%	0*
36	296	29.5%	39.5%	-*
43	300	22.3%	34.5%	-*
50	320	10.0%	28.7%	4.4
57	326	19.6	ND	--

^aExpressed as percent specific ⁵¹Cr release from the labeled platelets.

ND = not done.

*Autologous cytotoxicity was complicated by low platelet yield and tendency to clump during the acute phase of the disease.

Table 2. Relationship between platelet cytotoxicity^a and the indirect immunofluorescent antibody test (dog # 510).

Days PI	Isologous System		IFA Titer
	Lymphocytes Only	Lymphocytes and Serum	
0	12.9%	5.6%	0
5	25.2%	10.2%	1:10
15	32.0%	16.4%	1:320
22	30.0%	20.8%	ND
29	36.0%	45.6%	1:640
36	29.5%	39.5%	1:640
43	22.3%	34.5%	1:2560
50	10.0%	28.7%	ND
57	19.5%	ND	1:1280

^aExpressed as percent specific ⁵¹Cr release from the labeled platelets.

ND = not done.

D. The Effect of Serum and Macrophages Derived from *E. canis*-Infected Dogs on the *in vitro* Growth and Development of the Organism.

1. Establishment of a Canine Mononuclear Phagocytic Cell Tissue Culture Technique.

The canine mononuclear phagocytic cell (CMPC) *in vitro* cultivation technique developed by Nyindo *et al*⁷ and later modified by Huxsoll and Hemelt (unpublished) has been established, further modified, and refined in our laboratory. This CMPC system now provided for the continuous and reliable production of large quantities of *E. canis* antigen suitable for use in the IFA test for the detection of anti-*E. canis* antibodies.

Our CMPC culture technique employs the use of 6 normal dogs as donors for CMPC's and autologous serum with which to supplement the cells. Dogs are fasted for 12 hours before bleeding to reduce serum lipid levels. Approximately 300 ml of blood is collected from each dog every 4 to 6 weeks. Serum harvested from the whole blood is filtered first through a .45 μ then a .20 μ filter. Serum is divided into 10 ml aliquots and stored in sterile vials at - 20 C.

Every 3 to 4 weeks, 40 ml of whole blood from each of 2 dogs is collected in a 60 ml plastic syringe containing 10,000 units of Na heparin U.S.P. and 20 ml of 2.5% dextran (MW 500,000). Syringes are inverted (needle end upward), placed in a flask clamp, attached to a ring stand, and allowed to remain in this position for 45 minutes to 1 hour. A 5-ml aliquot of the leukocyte-rich supernate is then expelled from the syringe into 1 each of 6 to 8, 25 cm²

tissue culture flasks.* These flasks are placed in a dry 35 C incubator for 48 hours. After incubation, the supernate of each flask is poured off, the remaining attached cells are washed twice with HBSS and fed with 5 to 7 ml of MEM Eagle with Earle's BSS** containing 16.5% autologous serum and .8% L-glutamine*** (200 nM solution). The autologous serum is heated at 56 C for 30 minutes before adding to the culture. Every 2 to 4 days thereafter, the supernate in each flask is discarded and replaced with fresh media, serum and L-glutamine.

Seven to 10 days after planting, depending upon spreading, growth, and number of CMPC's per flask, each flask is inoculated with 1 ml of supernate fluid from E. canis-infected cultures. The infectious inoculum is collected from a flask inoculated 7 to 10 days earlier. In this manner 2 isolants of E. canis have been maintained in our laboratory for 33 (WRAIR) and 14 (Ill.) passages. The supernate used as inoculum usually consists of approximately 1,000 to 5,000 cells of which 59 to 95% contain at least one morula of E. canis. Every 2 to 4 days thereafter, the inoculated flasks are fed as described above. The percent cells in the supernatant of the inoculated flasks containing 1 or more morulae of E. canis are monitored daily after the 4th post inoculation day. One ml of supernate is collected from 2 of the 6 to 8 flasks and cells deposited by centrifugation on a 12-mm circular glass cover slip situated at the bottom of a cylindrical vial 14 mm in diameter.

*Falcon 3012 tissue culture flask, Oxnard, CA.

**Microbiological Associates, Bethesda, MD.

***Microbiological Associates, Bethesda, MD.

The vial is centrifuged at 600 g for 10 minutes thus forcing the cellular content of the supernate onto the cover slip.

The cover slip is removed, fixed in absolute methanol, Giemsa stained, mounted on a glass slide and examined to determine the percent cells containing 1 or more morulae of E. canis.

Between 7 to 12 days after inoculation, when the majority of attached cells in each flask have "rounded up" and 50 to 80% of the cells in the supernate contain at least 1 morula of E. canis, the attached and supernatant cells in each flask are harvested by the method of Nyindo et al.⁷ Approximately 27 canine sera can be screened with the infected cells harvested from 1 flask.

The modifications and refinements described above have allowed for the increased accuracy, timely reporting, and efficient screening of more than 1740, and titering of another 230, canine sera. This technique has provided for the production of antigen within the same and only laboratory performing the IFA test for canine ehrlichiosis.

2. Development of 24 Well Culture Plate Technique for the Growth and Study of CMPC's.

The closed, air-tight technique modified in our laboratory for the production of E. canis antigen did not allow for the sequential evaluation of viable attached CMPC's. Therefore an additional and different technique was needed.

A screw-cap Leighton tube technique was initially evaluated but was found insufficient due to the tendency of culture media to turn basic in pH (very toxic to CMPC's) within 12 to 24 hours after planting. This technique was not appraised in a CO₂ incubator,

with loosened tube caps, for fear of bacterial, fungal or viral contamination of the antibiotic-free CMPC cultures. Three 24 well tissue culture plates were evaluated. Into each 16 mm well was placed a circular 13 mm glass cover slip. One to 2 ml of leukocyte-rich supernatant was added to each well. A series of experiments were subsequently performed to evaluate the usefulness of such a technique for the culturing, staining, and sequential observation of E. canis-infected CMPC's maintained under a variety of conditions such as in the presence of normal or E. canis immune serum, immune serum fractions, normal and immune CMPC's.

Canine mononuclear phagocytic cells attached, spread, and matured satisfactorily in the 24 well tissue culture plate technique when maintained in a 5% CO₂ forced air incubator at 37 C. Bacterial and fungal contamination of cultures was rarely a significant problem. Of the 3 plates evaluated, Falcon,* Limbro,** and Costar,*** the Costar plate was found to be the most useful in our laboratory. The Costar plate technique has since been used in conjunction with flask-grown CMPC's to evaluate the effect of normal and immune (anti-E. canis) serum on the growth and development of E. canis in normal and carrier CMPC systems.

3. Investigation of the Effect of Immune and Normal Canine Serum on the Growth of E. canis within CMPC's Derived from Carrier and Normal Dogs.

*Falcon 3008 multiwell. Oxnard, CA.

**Limbro, multi-dish dispo-trays, Chemical Co., Inc., Hamden, CT.

***Costar, 3524 tissue culture cluster, Cambridge, MA.

Experiments using flask and tissue culture plate techniques were conducted to evaluate the effect of both immune and/or normal serum on the growth of E. canis within CMPC's derived from both carrier and normal animals. Figures listed in Table 1 for experiment # 1 represent the mean of 6 groups of CMPC cultures derived from 5 separate normal dogs. Six flasks per group were inoculated 7 to 10 days after planting with 1 ml of E. canis-infected supernate per flask. In each experiment listed in Tables 1 - 4, except where indicated differently, the figures listed under the days post inoculation % cells infected were arrived at as follows: Where values for only 1 day are listed, and where values are listed for more than 1 day, the last day's values only were determined by suspending attached cells in the supernatant and subsequently counting the total number of cells in the supernatant per 300 cells, containing 1 or more morulae of E. canis. The method used was the same as that described in the previous section for monitoring infected cultures. In the experiments where values are listed for more than 1 day, the determinations were made from the cells in 1 ml of supernatant per flask.

Numerous attempts were made to produce and subsequently freeze and store a "standard inoculum" of cell-free E. canis. Various methods of harvesting, centrifugation, and freeze-thaw techniques were employed. After many attempts at producing and freezing a standard inoculum with highly variable results, this effort was temporarily abandoned. Greatly reduced viability and loss of infectivity were consistent problems. Inoculum used for experiment #8, Table 3, was

twice frozen at - 20 C and thawed at 37 C immediately before use. However, in subsequent experiments (not listed in this report) the aliquoted frozen inoculum showed a great loss of infectivity.

Experiments 2 and 5, Table 1, were prepared to determine if morulae of E. canis could be detected in cells collected from 2 proven carrier dogs. The term carrier dog is used rather than immune dog because such dogs were proven by subinoculation techniques to be carriers of E. canis. However, it was not proven that these dogs were immune to E. canis. The carrier dogs had been infected by ticks 6 to 12 months before their use in this study. Sera from each dog exhibited an IFA E. canis specific titer greater than 1:2560.

Experiments 3 and 4 of the same table were designed to evaluate the effect of immune serum on the growth of E. canis in normal mononuclear phagocytic cells; to determine if cells from carrier dogs could be infected with E. canis, and to ascertain what influence normal and immune sera might have on the ability of carrier cells to become infected with, and support the growth of, E. canis.

In experiments 1, 3, and 4, the inoculum was added to each culture and the culture fed within 5 minutes with media containing 16.5% normal or immune serum. The carrier cells used in groups 2 and 3 of experiment 4 were derived from one carrier dog and the cells used in groups 4 and 5 from a second carrier.

Experiments 6 and 7, Table 2, were conducted to evaluate the necessity of allowing E. canis inoculum to adsorb to cultured cells for 1 to 2 hours prior to the addition of immune serum. The growth of E. canis in normal and carrier cells in the presence of normal

or immune serum were re-evaluated. The cells used in groups 4 to 6 and 7 and 8 were from 2 different carrier dogs.

Experiments 8-9, Table 3, were designed to determine the effect of incubating the inoculum with various dilutions of immune serum on the growth of E. canis in normal CMPC's. Since the inoculum for all of the experiments contained cell bound as well as cell free E. canis, the adsorption time for experiment 9 and subsequent experiments was extended to 2 days. This prolonged adsorption time would allow the ehrlichiae released from any inoculum cells which may have been trapped in the culture to be exposed to the serum in which the inoculum was incubated. The 10 or 30 minute incubation period prior to inoculation would, of course, expose the cell free ehrlichiae to the indicated serum prior to inoculation. Experiment 9 also represents the first attempt to estimate the number of E. canis infected cells per inoculant introduced into each flask in each group.

Experiments 10 and 11 in Table 4 were performed to compare the effect of fresh vs. heated immune and normal serum, when incubated with inoculum prior to a 2-day adsorption, on the growth of E. canis in both normal and carrier macrophages.

The objectives of experiment 12 were the same as those of 11 with the exception that the cultured cells were collected from carrier dogs (2). Cells from a normal dog served as a control on inoculum infectivity.

4. Results

The consistency with which normal CMPC's, maintained in normal serum, could be infected with and support the growth of E. canis

was established in the 6 groups of flasks used in experiment 1 (Table 1).

Morulae of E. canis were not detected in non-inoculated cells derived from carrier dogs in experiments 2 and 5 (Table 1).

The continual presence of immune serum before and after inoculation of normal cultures resulted in a noticeably lower percent, as compared to normal cell-normal serum system, of infected cells in experiment 3. Growth of E. canis in carrier cells ranged from 23% in those cells continuously in the presence of normal serum to nondetectable levels in those exposed to only autologous immune serum.

Experiment 4 revealed a somewhat similar suppression effect of immune serum, as shown in experiment 3, on the growth of E. canis in carrier-derived cells. However, the presence of immune serum did not completely retard the growth of E. canis in carrier cells.

In experiment 6, normal cells maintained in immune serum, after a 1-hour adsorption period, expressed a 55% infection rate at 10 days as compared to an 85% rate in cells maintained in normal serum (Table 2).

In experiment 7, the presence of immune serum before and/or after inoculation of cells again proved to retard the growth of E. canis in normal cells. Cells derived from each of 2 carrier dogs and continuously exposed to autologous immune serum showed a very low percentate, 4 to 7%. Carrier cells, from both dogs, raised and maintained in normal serum exhibited a higher percentage, 19 and 20%, of E. canis infected cells.

The effect of various dilutions of immune serum and prolonged serum-inoculum adsorption times on the growth of E. canis in normal CMPC's were explored in experiments 8 and 9 (Table 3). The 24 well tissue culture plate technique was used in experiment 8. Data from both experiments clearly revealed an increased percent of infected cells with sequential dilutions of immune serum.

Data from experiments 10 - 12 are presented in Table 4. Experiment 10 was employed to reveal the effect of fresh rather than heated immune serum on the growth of E. canis in normal cells. The percent cells containing morulae of E. canis clearly increased in direct correlation with decreasing concentrations of immune serum.

The effect of fresh or heat inactivated immune and normal serum on the growth of E. canis in mononuclear phagocytic cell culture derived from carrier and normal dogs is shown (Table 4). The presence of fresh immune serum seemed to more effectively suppress the numbers of infected cells as shown by 51% heated serum vs. 28% fresh serum infection rate. The presence of immune serum either as 10% or as 100% of the total serum volume (16.5%) in the inoculum resulted in a lowered percent of E. canis infected cells when compared to cells within the same experiment exposed only to normal serum.

The last experiment, # 12, in this study was designed to incorporate all of the adaptations made in the previous experiments into a single experiment in which the effect of immune vs. normal serum could be evaluated in cell cultures derived from carrier dogs. Carrier cells in continuous contact with only immune serum

and inoculated with inoculum incubated with immune serum did not exhibit E. canis at any of the 4 periods of evaluation. However, carrier cells maintained in normal serum exhibited 31 to 42% parasitemias. Normal cells raised and maintained in normal serum had a 73% final cell infection rate.

5. Summary and Comments

Several definite and repeatable but not unexpected patterns have become evident. Canine mononuclear phagocytic cells collected from E. canis carrier dogs and supplemented with autologous immune serum are substantially resistant to infection by, and/or intracellular growth of, E. canis.

Canine mononuclear phagocytic cells derived from normal dogs and supplemented with autologous serum are highly susceptible to infection by E. canis. Ehrlichia canis readily multiplies within such a system and destroys the monolayer within 7 to 14 days of inoculation.

Cells of normal dogs, supplemented with heated or fresh whole immune serum, resist infection to a greater degree than do normal cell-normal serum systems but they demonstrate a greater percent of infected cells than occurs in systems composed of carrier cells and normal serum.

These experiments were carried out in part during a time when our laboratory was establishing, adapting, and modifying the previously described tissue culture systems. Therefore, it was not always possible, as is reflected in the data, to have an identical number of either free ehrlichia or infected cells in the inoculum

used for each experiment. It must also be kept in mind that each group of cells used for each experiment expressed some degree of individual variation as to the number of cells per flask, as well as rate of spreading and apparent metabolic activity. Comparisons are thus best made only within a single experiment.

Table 1. Effect of immune and normal serum on the growth of *Ehrlichia canis* in CMPC's derived from carrier^a and normal dogs.

Exp. No.	System		Inoculum ^d % Cells Infected	Adsorption Time-Min.	Maintenance Serum	Days Post Inoculation % Cells Infected				
	Cells ^b	Serum ^c				7	10	14	18	21
1 ^e	N	N	73	5	N	51	73	87		
2	C	I	Not Inoculated		I	0			0	0
	C	N	Not Inoculated		N	0			0	0
3	N	N	75	5	N					82
	N	N	75	5	I					51
	N	I	75	5	I					37
	C	I	75	5	I					0
	C	N	75	5	N					23
	C	N	75	5	I					1
4	N	N	70	5	N	68				
	C	N	70	5	N	27				
	C	I	70	5	I	4				
	C	N	70	5	N	11				
	C	I	70	5	I	3				
5	C	I	Not Inoculated		N				0	0
	C	N	Not Inoculated		I				0	0

^aDogs proven by subinoculation of whole blood to be carriers of *E. canis*.

^bCells derived from normal = N, or carrier - C dogs.

^cSerum harvested from normal = N, or carrier dogs. Carrier dog serum considered to be immune due to *E. canis* specific IFA titer greater than 1:2,560.

^dInoculum was 1 ml of supernate of an *E. canis*-infected macrophage culture.

^eValues for experiment 1 are the mean of 6 groups of 5-6 flasks per group.

Table 2. Effect of immune and normal serum on the growth of *Ehrlichia canis* in CMPC's derived from carrier^a and normal dogs.

Exp. No.	System		Inoculum ^d % Cells Infected	Adsorption Time-Hr.	Maintenance Time	Days Post Inoculation	
	Cells ^b	Serum ^c				7	10
6	N	N	13	1	N		85
	N	N	13	1	I		55
7	N	N	68	2	N	64	
	N	I	68	2	N	18	
	N	I	68	2	I	18	
	C	I	68	2	I	5	
	C	I	68	2	N	4	
	C	N	68	2	N	19	
	C	I	68	2	I	7	
C	N	68	2	N	20		

^aDogs proven by subinoculation of whole blood to be carriers of *E. canis*.

^bCells derived from normal = N, or carrier = C dogs.

^cSerum harvested from normal = N, or carrier dogs. Carrier dog serum considered to be immune due to *E. canis* specific IFA titer greater than 1:2,560.

^dInoculum was 1 ml of supernate of an *E. canis*-infected macrophage culture.

Table 3. Effect of immune and normal serum and serum-inoculum incubation time, on the growth of *Ehrlichia canis* in CMPC's derived from normal dogs.

Exp. No.	System		% Cells Infected	Inoculum		Incubation Time-Min.	Adsorption Time-Hr.	Maintenance System	Days Post Inoculation		
	Cells ^a	Serum ^b		Infected Cells	Immune Serum %				% Cells Infected 5-7	9-10	11-13
8	N(TP)	N	15	250	10	2.5	N	2	4	3	
	N(TP)	N	15	100	10	2.5	N	3	4	5	
	N(TP)	N	15	50	10	2.5	N	3	9	13	
	N(TP)	N	15	25	10	2.5	N	10	13	22	
	N(TP)	N	15	12.5	10	2.5	N	13	30	30	
	N(TP)	N	15	0	10	2.5	N	23	41	70	
9	N	N	47	634	30	2 da	N	3	7	13	
	N	N	47	634	25	2	N	2	3	20	
	N	N	47	634	10	2	N	2	6	25	
	N	N	47	634	0	2	N	8	15	30	

^aCells derived from normal - N dogs.

^bSerum derived from normal = N dogs.

^cApproximate number of infected cells per inoculum.

^dExpressed as % of the serum used in culture media - 100% = 16.5% serum in the culture medium.

^eInoculum and serum containing culture media were incubated together prior to introduction into system.

TP = tissue culture plate coverslip method.

Table 4. Effect of immune and normal serum (heated vs. fresh), and serum-inoculation incubation time, on the growth of *Enrichia canis* in GIPC's derived from carriers^a and normal dogs.

Exp. No.	System		% Cells Infected	Inoculum		Incubation ^f Time-Min.	Adsorption Time-days	Maintenance Serum	Days Post Inoculation			
	Cells ^b	Serum ^c		Infected Cells	Immune ^e Serum				5-6	7-8	9-11	12-16
10	N	N	19	238	100(F)g	30	2	N	0	0	0	6
	N	N	19	238	25(F)	30	2	N	1	1	4	18
	N	N	19	238	10(F)	30	2	N	0	1	5	21
11	N	N	19	238	0(F)	30	2	N	1	3	10	56
	N	N	35	2,625	100	30	2	N	2	14	35	51
	N	N	35	2,625	100(F)	30	2	N	10	10	21	28
12	N	N	35	2,625	10	30	2	N	5	15	33	78
	N	N	35	2,625	10(F)	30	2	N	11	17	45	74
	N	N	35	2,625	0	30	2	N	26	42	71	96
12	N	N	35	2,625	0(F)	30	2	N	25	34	52	73
	C	I	53	675	100	30	2	I	0	0	0	0
	C	N	53	675	0	30	2	N	0	7	16	42
12	C	I	53	675	100	30	2	I	0	0	0	0
	C	N	53	675	0	30	2	N	0	2	14	31
	N	N	53	675	0	30	2	N	11	31	67	73

^aDogs proven by subinoculation of whole blood to be carriers of *E. canis*.

^bCells derived from normal = N, or carrier = C dogs.

^cSerum harvested from normal = N, or carrier = C carrier dog. Carrier dog serum considered to be immune = I, due to *E. canis* specific IFA greater than 1:2,560.

^dApproximate number of infected cells per inoculum.

^eExpressed as % of the serum used in culture media - 100% = 16.5% serum in the culture medium.

^fInoculum and serum containing culture media were incubated together prior to introduction into system.

^gFresh serum (F) - all other sera heat inactivated at 56 C for 30 minutes.

E. A Study of the Brown Dog Tick, *Rhipicephalus sanguineus*, as an Efficient Reservoir of *Ehrlichia canis*.

1. Introduction

The brown dog tick, *Rhipicephalus sanguineus*, was first incriminated as a vector of *E. canis* (*R. canis*) by Donatien and Lestoquard.² They infected susceptible dogs by inoculation with crushed *R. sanguineus* ticks harvested from dogs with acute canine ehrlichiosis. The brown dog tick has since been recognized as a vector of *E. canis*. Transmission of *E. canis* by ticks occurs transstadially but not transovarially.¹⁸ Information pertaining to the longevity of *E. canis* in the developmental stages of *R. sanguineus* and the efficiency of this tick in transmitting the organism after prolonged periods of fasting is unavailable. Thus, the importance of the unfed, *E. canis*-infected brown dog tick as an efficient natural reservoir of *E. canis* remains to be determined.

2. Materials and Methods

Eight mixed breed dogs, 10 months to 3 years of age, were used. Each dog had been vaccinated against canine distemper, infectious canine hepatitis, and leptospirosis within 1 year of use. Hematologic values of all dogs were within the normal ranges for the age and sex of each individual animal and each was seronegative for *E. canis* antibodies. Ticks were infected by allowing them to engorge on dogs in the acute phase of ehrlichiosis using the screw-cap capsule

18. Groves, M. G., Dennis, G. L., Amyx, H. L. and Huxsoll, D. L. Transmission of *Ehrlichia canis* to Dogs by Ticks (*Rhipicephalus sanguineus*). Am. J. Vet. Res. 36 (937-940).

method.¹⁹ The Illinois isolate of E. canis was used.¹⁹

Infected dogs were examined daily. Thrombocyte and total leukocyte counts were made, rectal temperature was recorded, and Giemsa-stained peripheral blood buffy coat smears were examined every 2 to 3 days for 120 days and bi-weekly thereafter. Canine ehrlichiosis was confirmed by the observation of E. canis morulae within mononuclear cells of a buffy coat smear, a rectal temperature 39.2 C or higher, severe thrombocytopenia and a positive IFAT for E. canis antibodies.

3. Tick dissection. Viscera were dissected from infected ticks and stored in Eagle's MEM that contained 20% dog serum, according to the the method of Smith.²⁰
4. Experimental design. Approximately 150 noninfected larvae were fed on a dog in the acute phase of canine ehrlichiosis. After the larvae had engorged and detached they were allowed to moult to nymphs. The nymphs were fed on a second dog that had acute canine ehrlichiosis, collected after detaching and allowed to moult to adults (Table 1). The day of detachment of the last nymph was designated day 0. On days 44, 59, 65, 92, and 155 post detachment, 15 female and 9 male unengorged adults were selected from the pool of unfed adults and allowed to engorge on a normal dog (Table 1). After 4 days of feeding, 6 partially engorged adults, 3 females and 3 males, were

19. Smith, R. D., Small, E., Weisiger, R., Byerly, C. S. and Ristic, M. Isolation in Illinois of a Foreign Strain of Ehrlichia canis, the Causative Agent of Canine Ehrlichiosis (Tropical Canine Pancytopenia). JAVMA, 166 (1975): 172-174.

20. Smith, R. D., Sells, D. M., Stephenson, E. H., Ristic, M. and Huxsoll, D. L. Development of Ehrlichia canis, Causative Agent of Canine Ehrlichiosis in the Tick Rhipicephalus sanguineus and its Differentiation from Symbiotic Rickettsia. Am. M. Vet. Res. 37 (1976): 119-126.

removed from dog #101 (day 155 ticks). The ticks were dissected, and the pooled viscera intravenously inoculated into dog #19.

5. Results

The 5 dogs that had been fed upon by E. canis-infected adult ticks developed acute canine ehrlichiosis (Table 1) and the relative degree of severity of disease produced was essentially identical. The incubation period ranged from 10 to 15 days, with a mean of 12.6 days. Clinical signs of ehrlichiosis were observed with dog #19, which was inoculated with infected tick viscera, on day 11 post inoculation.

6. Discussion

Results of the experiment conclusively demonstrated the potential of R. sanguineus as a significant and possibly a principal natural reservoir of virulent E. canis. This organism was successfully transmitted during the feeding of infected adults upon susceptible dogs at 44, 59, 65, 92, and for as long as 155 days after the ticks, as engorged nymphs, had detached from an acutely infected dog. The 155-day feeding exhausted our infected adult tick pool, therefore, we were unable to evaluate the infectivity of ticks maintained beyond 155 days. However, the longevity of unfed R. sanguineus adults is reported to range from 158 to 568 days.²¹

A minimal longevity of only 155 days would, in most moderate climates, afford an obvious mode for both the vector and pathogen

21. U.S. Department of Agriculture/Agricultural Research Service. Manual on Livestock Ticks (1966): 72-74.

to over-winter and thus infest and infect new non-resistant canine hosts in the following spring.

Our data is not inconsistent with that of Price²² who identified virulent R. rickettsii in unfed adult Dermacentor variabilis vectors for over 8 months, and that of MacLeod²³ who reported the existence of virulent E. phagocytophilia in adult ticks 14 months after their initial infection.

Of all hard ticks, R. sanguineus is probably the most widely distributed hard tick species in the world. In addition to its habitat, this tick has the ability to survive and thrive in the often low-moisture environment of its preferred habitat, kennels and homes. Rhipicephalus sanguineus is the only species of hard tick in the United States that has developed a well-defined resistance to some acaricides.^{24,25}

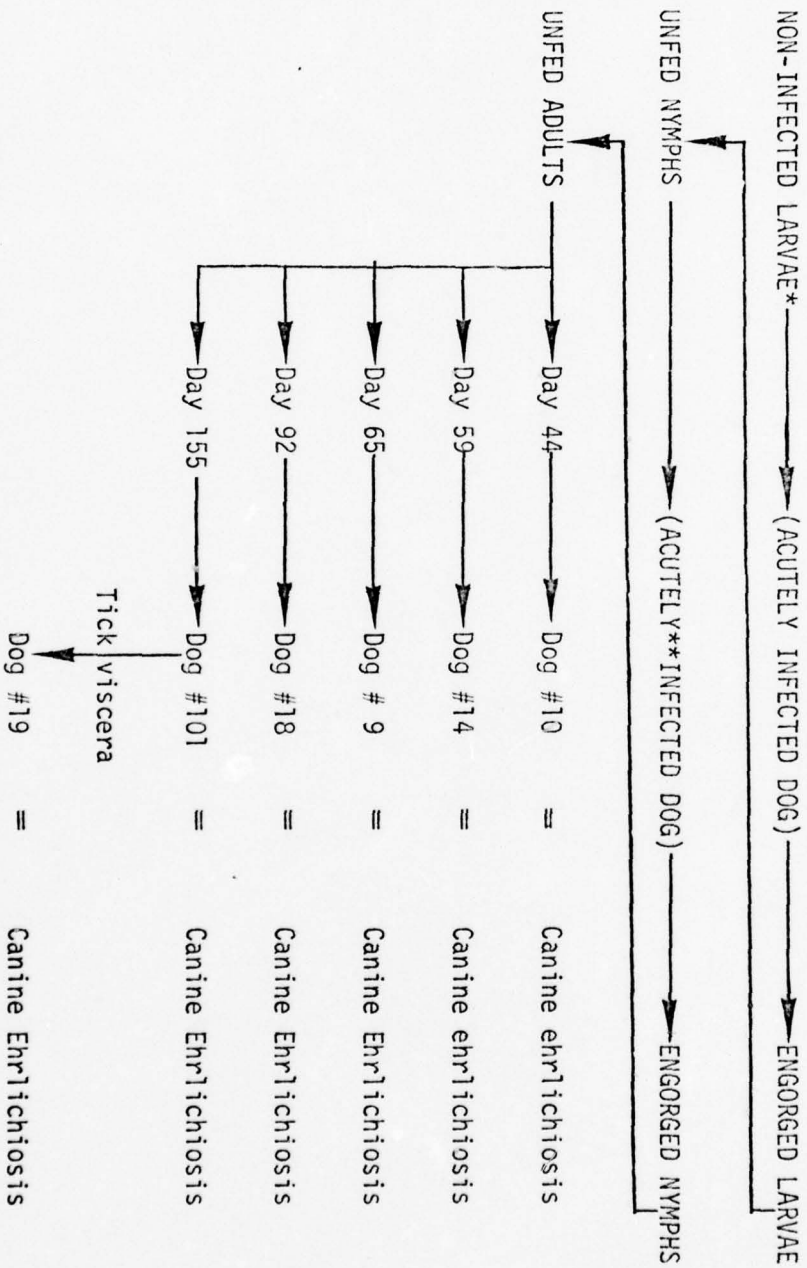
Thus the brown tick by virtue of its longevity, preferred habitat, principal host, polyphagia, adaptability, resistance to acaricides, and efficiency of transstadial transmission possesses the biological properties necessary to maintain stable environmental foci of E. canis, even when susceptible and/or carrier

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22. Price, W. H. The Epidemiology of Rocky Mountain Spotted Fever. II. Studies on the Biological Mechanism Rickettsia rickettsii. Am. J. Hyg. 60 (1954): 292-319.
 23. MacLeod, J. Studies in Tick-Borne Fever of Sheep. II. Experiments on Transmission and Distribution of the Disease. Parasitol. 28 (1936): 320-329.
 24. Graham, O. H. and Harris, R. L. Recent Developments in the Control of Some Arthropods of Public Health and Veterinary Importance. Livestock Insects. Bull. Entomol. Soc. Amer. 12 (1966): 319-325.
 25. Nelson, V. A. Human Parasitism by the Brown Dog Tick. J. Econ. Entomol. 62 (1969): 719-720.

animals are absent from the immediate environment for prolonged periods of time. It would seem feasible that because of the prolonged residence in the brown dog tick, E. canis, possibly other rickettsiae²⁶ could survive for extended periods, perhaps years, within an environment in which all dogs were prophylactically treated with tetracycline. The importance of R. sanguineus in the epidemiology and epizootiology of canine ehrlichiosis is strengthened by our demonstrating that this vector can serve as an efficient reservoir of E. canis.

26. Burgdorfer, W., Sexton, D. J., Gerloff, R. K., Anacker, R. L., Philip, R. N. and Thomas, L. A. Rhipicephalus sanguineus: Vector of a New Spotted Fever Group Rickettsia in the United States. *Infect. Immun.* 12 (1975): 705-210.

Table 1. Evaluation of unfed adult *Rhipicephalus sanguineus* ticks, which fed as larvae and nymphs on infected dogs, as reservoirs of *Ehrlichia canis*.



*Unfed *R. sanguineus* larvae, maintained for 2 previous generations on normal dogs.

**Acute canine ehrlichiosis; rectal temp - > 39.2 C, morulae of *E. canis* in peripheral blood monocytes, and severe thrombocytopenia.

Dogs #10, #14, #9, #18, #101 each developed acute canine ehrlichiosis within 10 to 15 days after exposure to the unfed *E. canis*-infected adult ticks.

F. Evaluation of the Dog Infected with *E. canis* as an Efficient Reservoir of Ehrlichiosis.

1. Introduction

Morulae of *E. canis* are readily demonstrable in the mononuclear cells of Giemsa-stained peripheral blood buffy coat smears prepared from dogs experiencing acute canine ehrlichiosis. However, morulae of ehrlichia are rarely observed in similar preparations from dogs recovered from the acute disease. Recovered dogs have been shown, by means of subinoculation of moderate amounts of whole peripheral blood, to remain carriers of virulent *E. canis* organisms for prolonged periods of time. These same carriers usually possess moderate to very high IFA test titers for specific anti-ehrlichial antibodies. Although dogs recovered from acute disease have repeatedly and consistently been shown by artificial means (mechanical subinoculation of whole blood) to harbor virulent *E. canis*, the ability of a carrier dog to serve as an efficient reservoir of significant numbers of virulent *E. canis* for engorging ticks has not been established.

2. Objective

To determine the potential of the *E. canis* carrier dog as an efficient reservoir of ehrlichiae for engorging *R. sanguineus* nymphs.

3. Materials and Methods

Nine (same as in Section C through and including tick dissection).

4. Experimental Design

Dog # 101 was used as the feeding source for 4 groups of 50 each uninfected, unengorged *B. sanguineus* nymphs. One group of

50 nymphs each was fed for 5 days starting 15, 25, and 41 days, respectively, after the dog was first exposed to E. canis-infected R. sanguineus ticks. A fourth group of 50 nymphs was allowed to feed for 10 days commencing 70 days post exposure. After each group had fed, the nymphs were allowed to moult to adults.

Twenty female and 12 male ticks were selected from the group of ticks which commenced feeding on dog #101, at 15 days post exposure, and allowed to engorge on dog #260 (Table 1). Twenty female and 12 male ticks were selected from each of the other 3 groups of ticks that had fed on dog #101. Each of these groups was allowed to engorge on a separate dog (Table 1). Four days after the adult ticks had attached, a total of 8 female and 6 male partially engorged ticks were removed from each of the following dogs: #260, #280, #247, and #248. All ticks from each dog were dissected and the viscera were pooled and injected intravenously into a susceptible dog (Table 1). Ticks remaining on each dog were allowed to complete engorgement and detach. Approximately 60 to 80 days after the ticks had detached, dogs #280, #247, and #248 were inoculated intravenously with 10 ml of E. canis-infected blood from dog #101. Ten ml of blood from dog #101 also was given intravenously to dogs #270, #103, and #250 70 days post inoculation of tick viscera.

4. Results

Of the adult ticks that had engorged on nymphs on dog #101, only those ticks that fed while the dog was in the acute phase of ehrlichiosis were capable of transmitting ehrlichiae (Table 1).

Dog #260 developed acute canine ehrlichiosis, as did dog #250 that was inoculated with pooled viscera from the same group of ticks that had partially engorged on dog #260. Each of the dogs that did not develop clinical signs of ehrlichiosis remained seronegative from E. canis antibodies. Subsequent challenge of the seronegative dogs with 10 ml of whole blood from dog #101 resulted in each dog developing acute ehrlichiosis indicating that each was susceptible to infection (Table 1).

5. Discussion

Results of this experiment indicate that a dog chronically infected with E. canis may not be an efficient and continuous reservoir of E. canis for engorging R. sanguineus nymphs. Only those ticks which engorged while dog #101 was parasitemic and experiencing acute ehrlichiosis, transmitted E. canis in a quantity and/or quality sufficient to produce acute disease in a susceptible canine host.

The removal of partially engorged adult ticks, the dissection of their viscera and its injection into susceptible dogs was carried out in an attempt to determine if adults which had engorged during the previous instar upon a chronically infected dog were harboring virulent E. canis organisms. It was speculated that such ticks may have acquired E. canis during engorgement on carrier dog #101 but were unable to subsequently transmit the disease. The results of visceral injections were identical to those obtained from the dogs on which a portion of the ticks from each group were allowed to complete engorgement. The only dog that developed acute canine ehrlichiosis or a positive IFA test

titer was dog #250. This dog was inoculated with the viscera of a portion of the tick group which engorged on dog #101 during the period of acute canine ehrlichiosis, day 15-20, while morulae of E. canis were readily observable in peripheral blood buffy coat preparations.

Donatien and Lestoquard² demonstrated by crushed tick inoculation that R. sanguineus can harbor E. canis after engorging on a dog with acute canine ehrlichiosis. They suggested that transstadial as well as transovarial transmission of E. canis occurred in the brown dog tick. The efficiency of this tick as a vector, after engorging in a previous instar on a dog parasitemic with E. canis has since been established. Transstadial transmission has been reported by Groves¹⁸ and Smith.²⁰ The previous report of Ewing and Philip.²⁷ Groves et al¹⁸ questioned the occurrence of transovarial transmission of E. canis in R. sanguineus. Similarly, transstadial but not transovarial transmission was demonstrated in Ixodis ricinus tick vectors of E. phagocytophilia, the causative agent of bovine and ovine tick-borne fever.^{23,28,29}

If, indeed, ehrlichial agents are not transmitted transovarially from an infected female to her progeny, then, as has been suggested by some investigators, the chronically infected dog would seem the likely environmental reservoir for E. canis. However,

27. Ewing, S. A., Philip, C. B. Ehrlichia-Like Rickettsiosis in Dogs in Oklahoma and its Relationship to Neorickettsia helminthoeca. Am. J. Vet. Res. 36 (1966): 937-940.
28. MacLeod, J. and Gordon, W. S. Studies in Tick-Borne Fever of Sheep. I. Transmission by the Tick, Ixodes ricinus with a Description of the Disease Produced. Parasitology, 25 (1933): 273-283.
29. Tuomi, J. Studies in Epidemiology of Bovine Tick-Borne Fever in Finland and a Clinical Description of Field Cases. Ann. Exp. Finn. 44 Supp. 6(1966): 1-62.

in view of our experimental results, the importance of the chronically infected dog as an efficient reservoir from which virulent ehrlichiae in a quantity and/or of a quality sufficient to produce disease may be drawn by natural tick feeding, rather than by experimental mechanical transmission of whole blood, is questioned.

The existence of virulent E. canis organisms within peripheral blood of dog #101 well after the remission of acute canine ehrlichiosis, and after completion of engorgement by the day 75-80 ticks, was established by challenging dogs #11, #13, #280, #247, #248, #270, #103 and #250, each with 10 ml of fresh whole blood collected from dog #101. Each of these 8 dogs developed acute ehrlichiosis within 2 weeks of challenge. We have since transmitted E. canis from dog #101 by subinoculation on 2 separate occasions. The possibility exists that the quantity of virulent E. canis organisms present in the peripheral blood of a dog recovered from acute ehrlichiosis may fluctuate considerably. However, if such a phenomena exists, it seems unlikely that we, on 3 consecutive occasions (days 25-29, 41-45, and 75-80), selected periods of low parasitemia to feed unengorged nymphs on dog #101. Possibly low numbers of ehrlichia and the presence of E. canis specific antibodies in peripheral blood ingested by ticks engorging on a chronically infected dog prevent infection of feeding ticks. These same factors may also inhibit survival within the ticks, or eventual transmission by the ticks, of E. canis.

88 Table 1. Evaluation of a dog infected with Ehrlichia canis as an efficient reservoir of ehrlichiae for engorging Rhipicephalus sanguineus nymphs.

Engorgement period of nymphs* (days)	Adult tick engorgement***		Challenge by infected blood****		Inoculated with adult tick viscera*****		Challenge by infected blood****	
	Dog No.	Developed ehrlichiosis	Developed ehrlichiosis	Developed ehrlichiosis	Dog No.	Developed ehrlichiosis	Developed ehrlichiosis	Developed ehrlichiosis
15-20**	260	yes	ND	ND	250	yes	ND	ND
25-29	280	no	yes	yes	270	no	yes	yes
41-45	247	no	yes	yes	103	no	yes	yes
75-80	248	no	yes	yes	250	no	yes	yes

*Approximately 50 R. sanguineus nymphs engorged during each time period on dog #101, which had been exposed on day 0 to E. canis by engorgement of E. canis-infected adult R. sanguineus ticks.

**Dog #101 was in the acute phase of ehrlichiosis.

***20 female and 12 male adult ticks selected from each group of nymphs that engorged on dog #101.

****Ten ml of E. canis-infected blood was obtained from dog #101 and used to challenge each dog.

*****Eight female and 6 male ticks were removed 4 days after attachment and dissected; viscera were pooled and injected intravenously into a susceptible dog.

ND = not done.

G. Ultrastructural and Biologic Properties of Ehrlichia equi.

1. Microscopic and Ultrastructural Features. When the buffy coat from horses in the acute phase of infection was stained by the Giemsa method, organisms occurring freely or as components of inclusion bodies were frequently found in the cytoplasm of neutrophils and eosinophils. They appeared oval to rod shaped and stained dark blue to purple. The organisms were detected by the electron microscope technique within membrane-lined vacuoles in the cytoplasm of neutrophils and eosinophils. Individual organisms were bound by 2 distinct membranes with the outer membrane, or cell wall, appearing rippled. The inner cytoplasmic membrane appeared adherent to electron-dense and lucid cytoplasmic constituents. The organisms varied in size from 0.2 μ to 1.5 μ but the internal structure remained similar. Static evidence of various stages or organismal division by binary fission was frequently in evidence.

Ehrlichia equi differed from classic rickettsiae in that the latter are usually more rod shaped and do not occur within membrane-lined vacuoles. It differs also from the organisms of the genus Chlamydia on the grounds that the developmental cycle consisting of elementary and intermediate bodies typical of the members of this genus was not in evidence. Static evidence of the organism entering or leaving host cells was not observed. It seems likely, however, that the entrance was by a phagocytic process and the exit of the organisms probably occurred as a result of rupture of vacuoles after the membrane of the vacuole and plasma membrane of the cell came in close apposition as has

been shown with other intracellular agents. It is concluded that morphologic characteristics of E. equi were consistent with agents of the genus Ehrlichia (see reprint by Sells et al.).

2. Host Range

Experimental inoculations with E. equi were attempted in cats, dogs, nonhuman primates, rats, mice, guinea pigs, hamsters and rabbits. The evidence of infection was based upon detection of morulae in the eosinophils and neutrophils of inoculated animals. Infected animals frequently showed accompanying hematologic signs of infection. Experimental infections were demonstrated in cats, dogs, and subhuman primates.

Morulae were observed in eosinophils of 1 of 3 cats inoculated with E. equi-infected canine blood. The organism was seen 7 and 8 days after inoculation.

Evidence of infection was produced in 11 of 17 beagle dogs and 3 of 6 German shepherd dogs inoculated with E. equi-infected canine blood. Intranuclear and intraeosinophilic morulae were detected 7 to 16 days after inoculation and persisted 1 to 4 days.

Six nonhuman primates (5 rhesus macaques and 1 baboon) were successfully infected with E. equi. All infected animals demonstrated morulae in their peripheral neutrophils 3 to 7 days after infection. Three rhesus macaques had pyrexia, increased sedimentation rate, and mild anemia. These abnormalities returned to normal one week after the disappearance of morulae. The susceptibility of nonhuman primates to infection with E. equi indicated the zoonotic potential of this agent.

3. Immune Responses

Both humoral- and cell-mediated immune responses in ponies infected with E. equi have been studied. An indirect fluorescent antibody (IFA) test was used for detection and titration of antibodies to E. equi. In all respects the test was similar to the IFA test for canine infections with E. canis.⁸ The antigen for E. equi, however, was not derived from cell cultures but rather from the blood of ponies during the acute phase of the disease. The antigen was prepared by applying buffy coat cells infected with E. equi onto a microscope slide.

For measurement of CMI responses, peripheral blood leukocytes were used as a source of sensitized lymphocytes in the LMI test. The antigen for the test was prepared by freezing and thawing 5 times in a dry ice-alcohol bath preparation of infected leukocytes derived from ponies during the time of acute infection.

After primary inoculation of 8 ponies with blood from another pony in the acute phase of infection, the animals developed signs of equine ehrlichiosis characterized by fever (107 F), anorexia, stiffness of legs, and marked thrombocytopenia. During the acute phase of infection, E. equi was demonstrated by the Giemsa and IFA staining methods in the cytoplasm of neutrophils and eosinophils. All 8 infected ponies became positive in the IFA and LMI tests by the 3rd week. In pony No. 31, antibody response reached a titer of 1:640 on day 25. On day 75 the titer was 1:280. There was a sudden drop of this titer after day 75 but again an increase was noted by day 225. The initial LMI response

was 50% at approximately 3 weeks after infection and gradually declined thereafter.

To ascertain whether pony No. 31 was a carrier of E. equi, the animal was euthanized and homogenates of blood, liver, spleen, and bone marrow were injected into 2 susceptible ponies. The latter animals failed to develop ehrlichiosis. They did, however, develop a transitory low titer IFA and LMI response. These animals developed a typical form of the disease when challenged with the blood of a pony in the acute phase of infection.

The data indicated that primary inoculation with blood of acutely infected ponies lead to clinical disease, and the recovered animals developed a state of apparently sterile protective immunity. Furthermore, the infection could not be transmitted by inoculation of blood and organ homogenates from recovered ponies or those which were subjected to systemic treatment with tetracycline. In view of the fact that the organism was transmitted only during the acute phase of the disease, this finding raises the question regarding the mode of natural transmission of E. equi.

H. Collaborative Studies with U.S. Army and Air Force Components.

Collaborative studies with scientists of various U.S. Army and Air Force installations on the control of canine ehrlichiosis and babesiosis by chemotherapy consisted of joint planning of experiments and conducting serologic tests to monitor the progress of control measures.

1. U.S. Army Medical Research Unit, Kuala Lumpur, Malaysia, c/o LTC David L. Huxsoll.

Prophylactic use of tetracycline against tropical canine pancytopenia in military dogs in Malaysia - Results of a 4-year study.

Introduction

Tropical canine pancytopenia (TCP), which has caused serious losses in military dog units, was first observed in Southeast Asia in 1963 in British military dogs in Singapore (Wilkins et al.)³⁰ The disease, characterized by fever, hemorrhage, edema, severe pancytopenia, and high mortality, was subsequently diagnosed in both military and privately owned dogs in Singapore and Malaysia (Spence et al.,³¹ MacVean, 1968³²). Tropical canine pancytopenia first appeared in this unit in 1964 when a severe outbreak which occurred over a 2-year period accounted for the death of numerous military dogs. An epizootic of TCP occurred in U.S. military dogs in Vietnam in 1968 and 1969 (Walker et al., 1970) and caused the

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30. Wilkins, J. H., Bowden, R. S. T. and Wilkinson, G. T. Vet. Rec., 81 (1967): 57-58.
31. Spence, J. B., Giam, C. H. and Theis, J. H. Vet. Rec., 81 (1967): 328-329.
32. MacVean, D. W. Malaysian Kennel Review, 66 (1968).
33. Walker, J. S., Rundquist, J. D., Taylor, R., Wilson, B. L., Andrews, M. R., Barck, J., Hogge, A. L. Jr., Huxsoll, D. L., Hildebrandt, P. K. and Nims, R. M. JAVMA, 157 (1970): 43-55.

death of approximately 200 dogs (Huxsoll et al.).³⁴ Outbreaks of the disease were often associated with heavy tick infestations (MacVean,³² Walker et al.,³³ Nims et al.³⁵).

The establishment of Ehrlichia canis as the causal organism of TCP followed detailed studies by an American team at the Walter Reed Army Institute of Research (Huxsoll et al.)³⁶ and in the United Kingdom by Seamer and Snape³⁷ at Porton. In subsequent studies the therapeutic and prophylactic value of broad spectrum antibiotics in treating and controlling this disease was demonstrated (Amyx et al.,³⁸ Seamer and Snape³⁹); E. canis grown in a cell culture system for the first time (Nyindo et al.);⁴⁰ an immunofluorescence test for serological diagnosis of TCP was developed (Ristic et al.)⁴¹ and Rhipicephalus sanguineus was shown to be an efficient vector of E. canis (Groves et al.).⁴²

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34. Huxsoll, D. L., Amyx, H. L., Hemelt, I. E., Hildebrandt, P. K., Nims, R. M. and Gochenour, W. S. Jr. *Exp. Parasitol.*, 31 (1972): 53-59.
 35. Nims, R. M., Ferguson, J. A., Walker, J. S., Hildebrandt, P. K., Huxsoll, D. L., Reardon, M. J., Varley, J. E., Kolaja, G. J., Watson, W. T., Shroyer, E. L., Elwell, P. A. and Vaeura, G. W. *JAVMA*, 158 (1971): 53-63.
 36. Huxsoll, D. L., Hildebrandt, P. K., Nims, R. M., Ferguson, J. S. and Walker, J. S. *Vet. Rec.*, 85 (1969): 587.
 37. Seamer, J. and Snape, T. *Vet. Rec.*, 86 (1970): 375.
 38. Amyx, H. L., Huxsoll, D. L., Zeiler, D. C. and Hildebrandt, P. K. *JAVMA*, 159 (1971): 1428-1432.
 39. Seamer, J. and Snape, T. *Res. Vet. Sci.*, 13 (1972): 307-314.
 40. Nyindo, M.B.A., Ristic, M., Huxsoll, D. L. and Smith, A. R. *Am. J. Vet. Res.*, 32 (1971): 1651-1658.
 41. Ristic, M., Huxsoll, D. L., Weisiger, R. M., Hildebrandt, P. K. and Nyindo, M.B.A. *Infect. Immun.*, 6 (1972): 226-231.
 42. Groves, M. G., Dennis, G. L., Amyx, H. L. and Huxsoll, D. L. *Am. J. Vet. Res.*, 36 (1975): 937-940.

Research responsibilities and methods

The present communication reports on the prophylactic use of tetracycline hydrochloride to control TCP in a military dog unit in Pulada, Malaysia, over a period of 4 years. Responsible investigator was British Major A. G. Wilder, MRCVS and the work was conducted in collaboration with the U.S. Army Medical Unit, Kuala Lumpur, Malaysia. The unit consisted of approximately 60 Labradors acquired from the United Kingdom, the United States and New Zealand. The tasks of the dogs necessitated their being in a jungle environment for both training and operations. Ticks were controlled by hand removal as part of the daily grooming routine and by dipping every 14 days in SEVIN carbaryl insecticide.* The dipping solution was prepared by mixing SEVIN 85 with water at the rate of 1 pound of active ingredient per 100 gal. water. This same solution was used to spray the permanent, concrete kennels which were used to house the dogs at base camp. The grass around the kennels was kept cut short. However, it must be appreciated that in this location absolute tick control was extremely difficult in working dogs, particularly those which were often used in lengthy training and operational exercises.

Following the report of Amyx et al.³⁸ which showed that dogs given tetracycline hydrochloride orally at a daily rate of 3 mg/lb body weight were refractory to infection with E. canis, a decision was made in 1972 to give each dog in the unit 1 250-mg capsule of tetracycline hydrochloride in its daily feed.

*Union Carbide, Salinas, CA).

Results

Since 1972 there has been no clinical evidence of infection in the unit. In addition, no untoward side effects from continuous administration of tetracycline have been observed, and no impairment of training or working capabilities of the dogs has occurred.

During the past 2 years a breeding program has been developed. Dogs in the breeding program are also given tetracycline prophylactically. There has been no impairment in conception, gestation, parturition, lactation, litter size or litter health.

Sera collected in 1972 from 22 dogs were tested in our laboratory for antibodies to E. canis by the indirect immunofluorescence (IFA) test. Antibody was demonstrated in 7 (32%) of the sera. Since E. canis has been shown to produce infections which persist in untreated dogs for many years (Ewing and Buckner,⁴³ and Groves et al⁴²) one can assume that many, if not all, of the dogs serologically positive in 1972 were indeed infected despite a lack of overt clinical signs. These findings also provided evidence that the organism was endemic in the areas in which the dogs were trained and utilized.

Serological studies on all dogs in the unit in November, 1974, showed that sera of only 2 dogs contained demonstrable antibody to E. canis and in both instances the titers were low (1:20). In follow-up studies in March, July and September, 1976, all dogs, including the 2 dogs which had demonstrable antibody in 1974, were found serologically negative on each of the 3 consecutive bleedings.

43. Ewing, S. A. and Buckner, R. G. Vet. Med. Small Anim. Clin., 60 (1965) 152-155.

Comments

Results of this study confirm the original finding at the Veterinary Division, Walter Reed Army Institute of Research, that tetracycline can be used to aid in the control of ehrlichiosis in military dogs. They also show that serologic monitoring by the IFA test can serve as an effective indicator of the progress of such a disease control program.

Since vaccines for E. canis are not available and control of ticks is extremely difficult in environments in which military dogs are often utilized, the prophylactic use of an antibiotic appears to be the only practical means at present for controlling the infection in a highly endemic area. The above study is the longest of its kind thus far conducted.

- b. Serologic recognition and clinical effects of babesiosis in dogs of the Malaysian military forces.

Clinical problem

In late 1972 and early 1973 a problem began developing in dogs of the Malaysian military forces. The dogs were trained and maintained at the Jungle Warfare School in Pulada. These dogs were assigned duties which would take them temporarily to areas as far as East Malaysia and the Thailand-Malaysia border, but they were returned to Pulada between assignments. The total population at Pulada fluctuated but was generally in the area of 50 dogs. With only a couple of exceptions the dogs were all of the Labrador breed.

The problem first surfaced because of the apparent loss of stamina that some dogs exhibited during the rigorous training exercises. A few dogs were found unable to complete the exercises.

On several occasions pale mucous membranes were noted, and packed cell volumes were found to be low in some dogs indicating a hematological cause of the problem. Canine babesiosis and/or ehrlichiosis were suspected but stained blood films did not reveal the presence of the causative agents.

Serologic diagnosis and hematologic findings

A serological survey using IFA tests for babesiosis and ehrlichiosis was performed on 14 November 1974 and at that time 8 of 43 samples submitted were positive for Babesia and 2 were positive for Ehrlichia. In March, 1975, an additional serological survey was performed on 45 dogs with 5 being found positive for Babesia and 2 for Ehrlichia. In August, 1975, an additional serological survey was performed on 25 dogs that were suspected either because of clinical observations or from the handlers' observations on stamina loss. Twelve of these dogs were found serologically positive for Babesia and none had demonstrable antibody to Ehrlichia.

Also in August, 1975, blood was drawn from 5 dogs exhibiting both lack of stamina and low packed cell volume. The heparinized blood was brought back to the Institute for Medical Research, Kuala Lumpur, under refrigeration, and each specimen was inoculated IV into a serologically negative dog. Two of the 5 inoculated dogs (#5 and #39) developed clinical signs of babesiosis with decreased packed cell volume (PCV) and hemoglobin (Hb). The PCV dropped to 17% on dog #5 and to 12% on dog #39, but then rose to above 30% by the end of October. Babesia gibsoni organisms were identified in Giemsa stained blood films from these 2 dogs. Four of the 5 inoculated dogs developed antibody to Babesia as indicated in Table I.

Additional hematological and serological examinations were performed on all available dogs at Pulada in March, 1976, July, 1976, and September, 1976. These findings, as well as those of previous group bleedings, are given in Table 2.

It should be noted that the August, 1975, bleeding was performed only on dogs having some evidence of loss of stamina and probably is not a true reflection of the total population. Additionally, it should be kept in mind that during this period of time many dogs were treated with Phenamidine (0.3 mg/kg, S.Q.), Diminazene acetate (Berenil) (3.5 to 7.0 mg/kg, IM) or Metromidazole (Flagyl) (200 mg/day, orally). Treatment was instituted primarily when dogs appeared to have lost stamina based on their performance in training exercises. The treatment presumably has had some effect on both the clinical appearance and the PCV levels.

Babesia gibsoni has been reported previously in Malaysia and it apparently is a rather common infection as evidenced by the recent observations that 6 of 16 serum samples from randomly selected young dogs were serologically positive for Babesia.

Comments

From the above observations it would appear that Babesia gibsoni infection is extremely difficult to diagnose solely on blood films. Repeated attempts to identify the organisms on smears from the Pulada dogs have met with failure, yet, passage of blood from 5 of the same Pulada dogs into 5 normal healthy dogs resulted in serological conversion in 4 of these dogs and clinical signs with demonstration of organisms on blood films in 2 dogs. The clinical history, hematological findings and serological evidence should all be combined to aid in diagnosis.

Babesiosis may go undetected in dogs not subjected to the stress of exercise. It became evident in military dogs at Pulada only after personnel extremely familiar with the physical capabilities of each dog detected a decrease in expected performance levels during rigorous training exercises. The importance of the disease in military dogs is obvious as it relates directly to their duty performance. In pet dogs the disease may go unnoticed, especially if not detected during the acute phase or if the acute phase is not severe. Therefore, Babesia gibsoni may be highly endemic in an area and yet be seldom diagnosed.

2. Division of Veterinary Resources, WRAIR, WRAMC: Veterinary Corps of the U.S. Air Force; Medical Component SEATO Laboratory, Bangkok, Thailand, c/o LTC Edward H. Stephenson.

LTC Stephenson is serving as coordinator of all domestic and overseas programs in which this laboratory participates. During the past year, more than 900 sera of dogs from the U.S. were examined for E. canis antibodies for the IFA test. Two smaller outbreaks of ehrlichiosis were diagnosed in Florida and Georgia. One of the reports describing canine ehrlichiosis in Florida⁴⁴ stated, "The cases presented in this report illustrate the importance of including canine ehrlichiosis (tropical canine pancytopenia) in the differential diagnosis wherever anemia, leukopenia, and thrombocytopenia are seen." The use of the IFA test has prompted awareness of ehrlichiosis among practicing veterinarians with the disease having been recognized in nearly all states where the vector tick exists.

The study of a large epizootic of canine ehrlichiosis in the Phoenix, Arizona area, under the direction of LTC Stephenson has

44. Harper, B. E. Vet. Med. Small Anim. Clin., (1975): 1153-1157.

been completed. The purposes of this study were to (1) define the extent of the epizootic of canine ehrlichiosis that occurred around Phoenix, (2) evaluate the number of clinical vs sub-clinical infections, (3) isolate the causative strain of E. canis associated with the epizootic, and (4) delineate an enzootic area from which purchase of dogs for military use may be made only after appropriate evaluation.

Antibodies to E. canis were detected by the IFA test in serums from 40 of 339 dogs (12%). The incidence rate was highest in German shepherd dogs (21%) as compared to 8% in the remaining breeds. A significant difference in disease rates between sexes was not observed. Dogs 12 weeks to 13 years of age were seropositive. The causative agent was isolated by inoculation of whole blood from a sero-positive dog into a susceptible German shepherd dog.

Detailed results of the IFA test among all breeds of dogs studied with differential diagnosis between German shepherd and non-German shepherd dogs representing working groups are given in Tables 3 and 4.

Results of the study by Davidson et al (reprint enclosed) describing use of tetracycline and the IFA test to monitor chemotherapeutic effects during an epizootic involving 315 dogs has been completed. On the basis of clinical and hematologic observations and results of the IFA test, the authors of the above paper concluded that canine ehrlichiosis is endemic throughout Thailand, and that E. canis is probably widely distributed through the canine population, frequently in a clinically silent form. These data suggest a need for special preventive and therapeutic measures to be taken with pet and working dog populations in Thailand to control ehrlichiosis.

The following is the list of Air Force bases from which canine sera for diagnosis of ehrlichiosis by the IFA test were received: Clark, Kadena, Osan, Aderson, Lackland, Davis, Mathan, Blytheville, Yokota and Udon. A total of 561 sera of individual dogs were screened for anti-E. canis and/or antibody titers determined. Seventy-two of these sera (128%) were positive for E. canis.

3. Division of Pathology, WRAIR, WRAMC, c/o LTC Paul K. Hildebrandt.

Definition of ultrastructural features of Ehrlichia equi was deemed necessary to further characterize it and compare it morphologically with E. canis and other rickettsial agents. Ehrlichia equi is the only antigenic relative of E. canis thus far found. Moreover, E. equi may have zoonotic potential in that experimental infections with it were induced in several animal species including monkeys. In a collaborative study with the Division of Pathology we have studied microscopic and ultrastructural features of E. equi. Detailed description of the results of this study are found in the enclosed reprint of a paper by Sells et al.

Table 1. Results of IFA test for *Babesia* on dogs inoculated with the blood from 5 suspected field cases of babesiosis.

Date	Dog #5	Dog #37	Dog #39	Dog #40	Dog #41
8/18/75	N	N	N	N	N
9/10/75	P	N	P	P	N
9/17/75	P	N	P	P	N
9/24/75	P	N	P	P	N
10/1/75	P	N	P	P	P
10/9/75	P	N	P	P	P
10/15/75	P	N	P	P	P

Table 2. Hematologic and serologic examination of dogs in Pulada for evidence of babesiosis.

Date	No. Examined	PCV Below 35%	No. Positive for Babesia
11/74	43	0	8 (18.6%)
3/75	45	7 (15.6%)	5 (11.1%)
8/75	25	12 (44.0%)	9 (36.0%)
3/76	52	13 (25.0%)	10 (19.4%)
7/76	49	7 (14.3%)	5 (10.2%)
9/76	48	2 (4.1%)	11 (22.9%)

Table 3. *Ehrlichia canis* antibodies revealed by the IFA test in serums from dogs in the Phoenix, Arizona vicinity.

Breed Group	Suspect Ehrlichiosis*		Non-suspect Ehrlichiosis**		Total	
Hound	3/11+	(27.3)++	2/29	(6.9)	5/40	(12.5)
Non-sporting	1/2	(50.0)	0/4	(0.0)	1/6	(16.7)
Sporting	5/13	(38.5)	0/55	(0.0)	5/68	(7.4)
Terrier	2/12	(16.7)	1/19	(5.3)	3/31	(9.7)
Toy	0/4	(0.0)	2/21	(9.5)	2/25	(8.0)
Working	15/62	(24.2)	7/100	(7.0)	22/162	(13.6)
Mix, unknown origin	0/1	(0.0)	2/6	(33.3)	2/7	(28.6)
Total	26/105	(24.8)	14/234	(6.0)	40/339	(11.8)

Table 4. *Ehrlichia canis* antibodies revealed by the IFA test in serums from working dogs in the Phoenix, Arizona vicinity.

Working group less German shepherd and German shepherd mix	1/21+	(4.8)++	0/40	(0.0)	1/61	(1.6)
German shepherd and German shepherd mix	14/41	(34.1)	7/60	(11.7)	21/101	(20.8)
Total working group	15/62	(24.2)	7/100	(7.0)	22/162	(13.6)

*Serums obtained from dogs that had experienced 1 or more episodes of epistaxis or had other signs consistent with canine ehrlichiosis.

**Serums obtained from dogs without a medical history that suggested ehrlichial infection.

+Number serologically positive/total tested.

++Percent serologically positive.

IV. SUMMARY OF PROGRESS DURING THE YEAR 1976

A. Immunopathologic Studies on Ehrlichiosis

A platelet migration inhibition (PMI) test was developed for detection of serum antiplatelet activity in experimentally and naturally induced canine ehrlichiosis. Maximum inhibition of platelet migration was caused by serum collected during the acute phase of the disease. Detectable quantities of PMI factor(s) in the serum preceded the appearance of specific antibody demonstrable by the IFA test and may be one of the earliest events leading to pancytopenia. In most cases, samples positive in the IFA test were also positive in the PMI test. No correlation was evident between the IFA titer and the degree of PMI. Inoculation of dogs with normal canine blood did not induce PMI or IFA activity.

Fractionation of normal preinfection and post infection serum by the gel filtration technique (Sephadex G-200) showed the greatest degree of PMI (67%) in the IgG peak followed by 31% in the IgM peak. Only 10.5% inhibition was observed in the albumin rich peak. Normal and preinfection sera showed 3 to 11.5% inhibition in the IgG-rich peak and no inhibitory activity in the IgM and albumin peaks. Preliminary studies showed that most of the PMI activity can be absorbed by incubating serum with normal canine platelets at 22 C for 1 hour.

Examination by scanning electron microscopy (SEM) of platelets treated with normal serum and serum having inhibitory activity revealed that uninhibited platelets possess numerous pseudopod formations whereas inhibited platelets are generally rounded, smooth, and occasionally have membrane damage and apparent shrinkage and

loss of intracellular contents. It was concluded that the PMI factor(s) interfere with migration by inhibiting pseudopod formation.

At the cellular level of immunopathologic studies, a test was developed showing that lymphocytes of dogs infected with E. canis exert a cytotoxic activity on ^{51}Cr labeled platelets. This activity was most pronounced during the acute phase of the disease. The addition of serum appeared to enhance the cytotoxic activity in some cases. Unlike platelet migration inhibition effect, the cytotoxic activity commenced at approximately the same time as immune response detectable by the IFA test.

B. Examination of Immune Responses to E. canis

To study the effect of serum and macrophages from infected dogs on growth and development of E. canis, 2 modifications of the original tissue culture technique were developed. In the first modification, which provides for continuous production of large quantities of E. canis antigen, canine mononuclear phagocytic cells were cultured with autologous serum used in the culture medium. The step eliminated histocompatibility effects between cells and sera of various dogs and allowed efficient examination of more than 1350, and titering of another 130, canine sera. In the second modification, a well-culture plate technique was developed which provided for sequential observation of E. canis-infected canine mononuclear phagocytic cells incubated under a variety of conditions to include the presence of normal or immune serum, fractions of immune serum, and normal and immune canine mononuclear phagocytic cells. By use of this technique, the following in vitro anti-E. canis immune

effects were revealed: (1) Canine mononuclear phagocytic cells derived from E. canis-free dogs and supplemented with autologous serum are highly susceptible to infection with the organism; (2) canine mononuclear phagocytic cells derived from E. canis-carrier dogs and supplemented with autologous immune serum are substantially resistant to infection by, and/or intracellular growth of, E. canis; (3) canine mononuclear phagocytic cells derived from E. canis-free dogs, supplemented with heated or fresh whole immune serum, resisted infection to a greater degree than did normal cell-normal serum systems, however, they demonstrated a greater percent of infected cells than systems composed of carrier cells and normal serum. Thus immune anti-E. canis systems as revealed by an in vitro method and cited in order of maximum to lowest activity are: immune cells-immune serum; immune cells-normal serum; normal cells-immune serum, and normal cells-normal serum.

C. The Brown Dog Tick, Rhipicephalus sanguineus as a Reservoir of Ehrlichia canis.

The potential of the brown dog tick, Rhipicephalus sanguineus as an efficient reservoir of E. canis was investigated. Unengorged R. sanguineus adults harbored and efficiently transmitted E. canis to susceptible dogs for as long as 155 days after detachment as engorged nymphs from a dog experiencing acute ehrlichiosis. The 155-day feeding exhausted our infected adult tick pool, therefore, we were unable to evaluate the infectivity maintained beyond 155 days. The longevity of unfed R. sanguineus adults is reported to last up to 568 days. The minimal longevity of only 155 days as shown in our study would, in most moderate climates, afford an obvious mode for

both the vector and pathogen to over-winter and thus infest new non-resistant canine hosts in the spring. The importance of R. sanguineus in the epizootiology of canine ehrlichiosis is further strengthened by the study.

D. Some Ultrastructural and Biological Characteristics of Ehrlichia equi.

Ehrlichia equi, the only rickettsia in North America found to be antigenically related to E. canis (1975 report), has been studied and some of its morphologic, infectious, and immunologic properties compared with those of E. canis. Unlike E. canis, the organisms occurs freely or as a component of inclusion bodies in the cytoplasm of neutrophils and eosinophils. Individual organisms were bound by 2 distinct membranes with the outer membrane, or cell wall, appearing rippled. Experimental host range of E. equi includes horses, dogs, cats and non-human primates. The susceptibility of nonhuman primates indicates the zoonotic potential of E. equi. Unlike canine infections with E. canis, horses recovered from primary infection with E. equi developed a state of apparently sterile protective immunity. We were unable to infect susceptible horses with blood and organ homogenates from horses following their recovery from the acute phase of the disease.

E. Collaborative Studies with the U.S. Army and Air Force Components.

Collaborative studies with scientists of various U.S. Army and Air Force installations on the control of canine ehrlichiosis and babesiosis by chemotherapy consisted of joint planning of experiments and conducting serologic tests to monitor the progress of control measures.

In Malaysia, U.S. Army Medical Research Unit, Kuala Lumpur, a 4-year study on prophylactic use of tetracycline against tropical canine pancytopenia in military dogs of that country was completed. In 1972 when the experiment started, 32% of the dogs had titer for E. canis; in 1974, the percent positive dogs was reduced to 10%, while in 1976 all dogs were negative for E. canis antibodies. Prolonged use of tetracycline did not cause impairment of conception, gestation, parturition, lactation, litter size or litter health of experimental dogs. The etiology of chronic anemia and lack of stamina among dogs (Labrador breed) of the Malaysian military forces was determined to be babesiosis caused by Babesia gibsoni. Clinically suspect cases were serologically positive for babesiosis and inoculation of blood from these animals into susceptible dogs showed that these sero-positive dogs are active babesia carriers.

Examination of sera of 561 dogs, belonging to 10 different Air Force bases, 72, or 12.8%, were found positive for E. canis antibodies. Serologic testing was designed to identify diseased dogs prior to relocation; secondly, disease control programs using continuous chemotherapy was initiated and monitored through serologic testing to determine the progress of such a program.

In the U.S., increased awareness of canine ehrlichiosis has been made by detecting serologically positive dogs in many states in which the vector tick is known to exist. The study of a large epizootic of ehrlichiosis among dogs in Phoenix, Arizona area has been completed. Antibodies to E. canis were detected in serums from 40 of 339 dogs (12%). The incidence rate was highest

in German shepherd dogs (21%) as compared to 8% in the remaining breeds. Dogs 12 weeks to 13 years of age were sero-positive. The causative agent was isolated by inoculation of whole blood from a sero-positive dog into a susceptible German shepherd dog. Since earlier purchases of dogs for military use were made from this area, it is recommended that future purchase be made only after careful serologic, hematologic and clinical examination of respective dogs.