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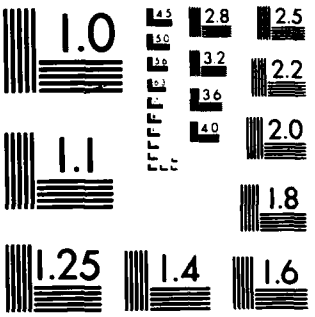

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

FINAL REPORT

October 1, 1969 - December 31, 1979

CONTROL OF HEMOTROPIC DISEASES OF DOGS

U.S. Army DADA 17-70-C-0044

by

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I. SUMMARY (1969-1979)

Only the more significant research contributions of the project toward solution of canine ehrlichiosis or tropical canine pancytopenia (TCP) will be cited. The development of the monocyte culture technique for *in vitro* propagation of the causative agent, the rickettsial *Erhlichia canis*, constituted an essential step toward successful studies of the agent and the disease. This technique provided for the first time a means of growing the organism in a system other than the dog, the natural mammalian host. Growth of the organism in monocyte cultures led to the development of antigens for serologic and immunologic studies. An indirect fluorescent antibody (IFA) test was developed and extensively used to detect *E. canis* infections among military dogs in and outside the United States and to monitor preventive and therapeutic measures for control of the disease. The antigen generated by the cell cultures also proved to be effective for *in vitro* tests of cell-mediated immunity (CMI).

The development of these techniques permitted further elucidation of the pathogenesis of TCP. The presence of the platelet migration inhibition factor (PMIF) was demonstrated in the serum of dogs naturally and experimentally infected with *E. canis*. The presence and the concentration of PMIF was measured by the platelet migration inhibition test (PMIT) and in the process, a correlation between the concentration of PMIF and severity of thrombocytopenia was revealed. In addition, knowledge of the immune response made possible the establishment of guidelines in formulating investigation on the control of the disease in military dogs. Collaborative laboratory and field studies with various U.S. Army units in Southeast Asia have shown that low daily doses of

tetracycline offers a means of controlling the disease in military dogs in an endemic area. The practice is gaining wide acceptance in areas where the maintenance of operational military dog units is dependent upon the control of ehrlichiosis.

Another important result of the collaborative research came from studies of the transmission of E. canis by its tick vector. It was shown that Rhipicephalus sanguineus is an efficient vector of E. canis and that transmission occurs transstadially but not transovarially. More recent studies demonstrated that the infectious agent can be maintained by the vector for a period of many months.

II. DETAILED REPORT

A. In vitro Cultivation of Ehrlichia canis

Ehrlichia canis, causative agent of tropical canine pancytopenia (TCP), has been propagated in monocyte cell cultures derived from the blood of dogs acutely infected with this agent. Tissue culture medium consisted of Eagle's minimum essential medium supplemented with 20% canine serum. Results of microscopic examination of monolayers stained by Giemsa and fluorescent antibody (FA) methods indicated that the intracytoplasmic ehrlichia underwent a specific cycle of development. Principal developmental forms were elementary bodies (individual ehrlichia organisms), initial bodies (immature organismal inclusions), and morulae (mature organismal inclusions). Five dogs each inolucated with 2- to 8-ml. volumes of cell culture suspensions harvested on days 5, 7, 20, 23, and 28 of incubation developed signs of TCP. The organism was reisolated from these dogs.

Because developmental cycle of ehrlichia demonstrated in the present study closely resembled that of the agents belonging to psittacosis-lymphogranuloma venereum (PLV) group of agents, and because the latter agents have also been found in ticks, reclassification of the agent from family Rickettsiaceae to family Chlamydiaceae has been suggested.

This work is considered a significant prerequisite for assays of fundamental properties of the organism, and it has all the essentials which would allow development of immunizing and diagnostic reagents for TCP.

B. Serologic Test for Canine Ehrlichiosis

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

C. Kinetics of Antibody Response to Ehrlichia canis

The kinetics of antibody production response to experimentally induced infection of dogs with Ehrlichia canis was determined by

ion-exchange and molecularsieve chromatography and by indirect fluorescent antibody (IFA) test. The first IFA antibody at 7 days after inoculation resided in immunoglobulin M (IgM) and immunoglobulin A (IgA) classes. At approximately 21 days after inoculation, the antibody was in IgM, IgA, and immunoglobulin G (IgG) classes. Thereafter, antibody concentrations continued to increase in the IgG class; those in the other 2 immunoglobulin classes had a variable pattern. In 2 dogs which died 60 and 114 days after inoculation, a decrease of antibody concentration in the 3 immunoglobulin classes was evident at the time of death. In the carrier dog, however, which was killed 147 days after inoculation, antibody concentrations sustained increasing titers in the 3 immunoglobulin classes.

D. Development of E. canis in Rhipicephalus sanguineus Tick

Certain aspects of the development of Ehrlichia canis, causative agent of canine ehrlichiosis (tropical canine pancytopenia), in Rhipiciphalus sanguineus ticks were studied. It was found that partial feeding of nymphs infected as larvae with E. canis was a desirable, if not necessary, preliminary treatment for successful infection of dogs with ground-up ticks. It remains unclear whether feeding increased the number or altered the virulence of ehrlichiae within tick tissues.

Ehrlichia canis organisms were detected by immunofluorescent microscopy in the midgut and hemocytes and by electron microscopy in the midgut and salivary glands of partially engorged adult ticks which had been infected as larvae and nymphs. Organisms were not

observed in the ovary. Intracytoplasmic inclusions contained 1 to 80 elementary bodies, each provided with 2 distinct membranes. Infection of the midgut and salivary gland was confirmed by injecting homogenates of these tissues into susceptible dogs. Staining of gut smears of partially engorged adult ticks by fluorescein-conjugated anti-E. canis antibody was found to be a reliable indicator of the infection.

Tick transmission of Ehrlichia canis, causative agent of canine ehrlichiosis (tropical canine pancytopenia), with the brown dog tick Rhipicephalus sanguineus has recently been demonstrated. Ticks were found to acquire infection as larvae or nymphs and to transmit E. canis transstadially. In contrast to earlier reports, transovarial transmission did not occur.

The results of light, fluorescent, and electron microscopic studies of the development of E. canis in the tissues of R. sanguineus are reported herein. Specifically, this study was designed to identify the sites of ehrlichial multiplication in the tick, characterize the forms morphologically, determine the possible mode of transmission to dogs, and compare findings with those reported for other tickborne rickettsiae. Certain biologic properties of the organism in infected ticks also were examined.

E. Platlet Migration Factor in Sera of Dogs Infected with E. canis

A platelet migration inhibition test was devised to determine the presence of antiplatelet activity in serum collected from experimentally produced and natural cases of canine ehrlichiosis. The maximum platelet

migration inhibition effect was observed during the acute phase of the disease and before the appearance of specific humoral antibody, measured by the indirect fluorescent-antibody test. Platelet migration inhibition may be one of the earliest events leading to pancytopenia. In most cases, sera positive for humoral antibodies also were positive for platelet migration inhibition, although no direct correlation was evident between the serological titer and the degree of platelet migration inhibition. Inoculation of dogs with uninfected canine blood did not induce the production of inhibition factor or antibody activity, which precluded a histocompatibility response to the cellular elements in the inoculum. Scanning electron microscopy indicated that the platelet inhibition factor interfered with platelet migration by inhibiting pseudopod formation. Affected platelets became rounded and showed evidence of clumping and leakage.

F. Cell-Mediated and Humoral Responses to Ehrlichia canis

Immunity to many infective agents is known to involve more than specific antibody interaction with the invading organism. Development of protection against various intracellular parasites of viral, rickettsial, bacterial and protozoan nature has been reported to be related to cell-mediated immunity (CMI). Recent reports have indicated the presence of CMI in anaplasmosis. The cell-mediated response has been studied with respect to various Anaplasma immunogens, including virulent and attenuated A. marginale in live and inactivated forms.

Cattle inoculated with virulent A. marginale had a marked and prolonged leukocyte migration inhibition test (LMIT) response which

began either early or late in the prepatent period. Cattle that received the attenuated A. marginale generally showed an early LMIT response after inoculation. The characteristically mild parasitaemia produced by the attenuated A. marginale causes no clinical disease, and the concomitant early cellular response may further moderate the infection. Animals given two doses of commerical vaccine (inactivated virulent organisms, bovine origin) had a transient, low-level LMIT response. Cattle that received the inactivated attenuated A. marginale of ovine origin responded as did the animals given the commercial vaccine. Susceptible cattle injected with virulent A. marginale provided information on the relationship of the CMI response to clinical illness. When the LMIT response was increased early in the prepatent period, the cattle survived the infection even though acute signs of anaplasmosis were evident. In situations where the LMIT response did not increase markedly until the end of the prepatent period or increased only after the onset of parasitaemia, the cattle became critically ill or died.

Lymphocyte-transformation studies indicated that the degree of sensitization of leukocytes reached much higher levels in cattle given inactivated A. marginale in adjuvant compared with those in cattle given viable A. marginale. The degree of increased cellular activity indicated by a degree of radioisotope incorporation, even in the absence of test antigen, was indicative of nonspecific stimulation of the reticulo-endothelial system possible caused by the use of an adjuvant-A. marginale preparation.

Recovery from acute virulent A. marginale infection was coincident with evidence of a strong CMI response and clinical protection against challenge inoculation. Cattle vaccinated with the attenuated A. marginale were also protected against challenge with virulent A. marginale. These findings indicate a correlation between CMI (measured by the LMIT) and protection. The CMI response seems to parallel antibacterial cellular immunity in the requirement of living cells to produce a continuous stimulus with concomitant residual sensitivity of leukocytes subject to an anamnestic secondary response. Complete protection against both parasitaemia and anaemia was demonstrated only in cattle which had clinically recovered from anaplasmosis or had been vaccinated with live attenuated A. marginale. Subsequent elimination of the carrier state has been reported to render the host susceptible to anaplasmosis, but this concept has recently been questioned, since cattle rendered free of Anaplasma organisms seemed immune to challenge inoculation. To further examine this observation, we subjected two four-year old cows, previously vaccinated with the attenuated agent and subsequently resisting challenge of the virulent organism, to systemic therapy. The animals received ten daily doses of oxytetracycline at 5 mg/lb administered intravenously. Subinoculation of 100 ml of whole blood from these cows into two splenectomized cows failed to produce infection in the recipient animals. Approximately 10 weeks after treatment the cows were rechallenged with virulent A. marginale. A prompt LMIT response was noted after the challenge and the animals demonstrated [illegible] clinical resistance.

G. The Effect of Tetracycline on E. canis Infection

Earlier studies showed that an oral continuous administration of tetracycline can be used therapeutically and prophylactically to control canine ehrlichiosis. A combination of such treatment with the use of indirect fluorescent antibody (IFA) test to monitor immune responses has produced excellent disease control results among military dogs in endemic areas. Based upon these earlier studies, several well-controlled experiments have been initiated by the U.S. Army Medical Unit in Kuala Lumpur, Malaysia, in collaboration with this laboratory to determine more exactly the effect of such treatment initiated during the various phases of the disease. The ultimate goal of these investigations is to develop a standard operational procedure (SOP) for control of ehrlichiosis in military dogs in endemic areas.

Experiments conducted during the past year were concerned with application of low level tetracycline (3 mg/lb/day), starting at 7 and 14 days after infection and continuing for 30 days. The treatment was successful regardless of the time (7 or 14 days after infection) when administration of tetracycline was initiated. Signs of the disease in dogs started on tetracycline 14 days post-inoculation disappeared rapidly after treatment was initiated. Results based on subinoculation of blood from infected to normal dogs during and after treatment showed that the treatment cleared all dogs of infection. Dogs cleared of infection, however, showed no protective immunity upon reinfection. Strong but transitory antibody responses were noted in all infected dogs regardless of when tetracycline therapy was initiated. Dogs which were reinfected at 60 days

after tetracycline treatment was discontinued, redeveloped antibody titers to E. canis. At 6 weeks after reinfection, the antibody titers, however, did not exceed titers measured in these animals as a result of the primary infection.

The isolation of E. canis from a dog in Negri Sembilan, Peninsular Malaysia, afforded an opportunity to study properties of the local strain. Mixed breeds of adult dogs were inoculated intravenously with this E. canis isolant. Inoculated dogs developed signs of the disease which included fever, weight loss, lymphodenopath, corneal opacity, and pancytopenia. Of 3 dogs that died during the course of the study, one died with severe pancytopenia 78 days post-inoculation, and hemorrhagic lesions were prominant in numerous organs. All inoculated dogs developed strong antibody titers to antigen prepared from a U.S. isolant of E. canis, indicating cross-serologic relationship between 2 isolants.

During the past year, 873 sera of dogs belonging primarily to the U.S. Armed Forces were examined for antibodies to E. canis by the IFA test; a total of 278 of these dogs were positive.

Serologic examination for babesiosis using the IFA test was made on 214 dogs belonging to the U.S. Armed Forces and allied armies. A total of 151 of these dogs were positive.

H. Relationship Between Ehrlichia canis and Rickettsia sennetsu

Similarities between R. sennetsu and E. canis thus far noted are their structural appearance in reference to host cells, their

specific growth cycle in peripheral blood monocyte cell cultures, and their serologic relationship using the indirect fluroescent antibody test (please see Progress Report IX, 1978). Unlike most other rickettsiae, E. canis and R. sennetsu occur in a membrane-like vacuole which separates individual organisms or groups of organisms from host cell cytoplasm. Various growth forms of the organisms observed in the sequence of their appearance in monocyte cell cultures were induvidual organisms in host cell cytoplasm, followed by formation of smaller and then larger inclusion bodies (morulae), individual organisms and inclusion bodies (morulae), individual organisms and inclusion bodies in large cytoplasmic vacuoles and, finally, the above-described growth forms occurring extra-cellularly.

Antigenic relationship between E. canis and R. sennetsu was demonstrated by reactivity of human sera of patients recovering from sennetsu rickettsiosis with cell culture-derived E. canis antigens using the indirect fluorescent antibody (IFA) test. In a preliminary study, canine sera of dogs infected with E. canis reacted with R. sennetsu antigen. The latter study is in progress in Japan.

Evidence of antigenic relationship between E. canis and R. sennetsu is a significant finding toward further definition of the finding is further amplified by the antigenic uniqueness of these two agents in reference to other rickettsiae. No antigenic relationship was demonstrated between R. sennetsu and Rickettsia

prowazeki, Rickettsia tsutsugamushi, Coxiella burneti, (Tanaka and Hanoaka, 1961), Rickettsia orientalis (Tachibana et al, 1976), Salmonellae (S. typhi, S. paratyphi A and B), Protens (OX 19, OX K, OX 2), Brucella suis, and Leptospiras (L. icterohaemorrhagiae, L. canicola, L. pyrogenes, L. grippotyphosa, L. hebdomadis, L. autumnalis, L. bataviae, L. javanica, L. pomona, L. australis A and L. mochtarii) (Misao and Kubayashi, 1954). Similarly, no cross-serologic relationship was demonstrated between E. canis and common bacterial and viral pathogens of dog as well as 8 rickettsiae (Ristic et al, 1972).

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