PATHOGENESIS OF TWO ISOLATES OF *EHRLICHIA CANIS* IN DOGS

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CHAPTER I

INTRODUCTION

Canine ehrlichiosis, caused by Ehrlichia canis (Donatien and Lestoquard 1935), was first recognized in the United States (Oklahoma) in 1962 (Ewing 1964a and 1964b) more than twenty-five years after its discovery in the Old World (Donatien and Lestoquard 1935). The 1962 Oklahoma strain of E. canis was found to be quite pathogenic for young pups and often produced a fatal disease under experimental conditions. E. canis occurs in the cytoplasm of leukocytes as a characteristic morula which is actually an aggregate of organisms. These morulae were found to parasitize lymphocytes primarily, monocytes less frequently and neutrophils rarely (Ewing 1964b).

The clinical manifestation and hematologic changes associated with canine ehrlichiosis were described by Ewing and Buckner (1965a). They concluded that the anemia produced by E. canis was of the normocytic-normochromic type. The clinical findings were similar to those described for combined Babesia-Ehrlichia infection. "...There was no clear differentiation between dogs with ehrlichiosis and dogs with combined infections of Ehrlichia and Babesia. Dogs with either condition developed severe anemia and apparently were unable to replace functional erythrocytes rapidly enough to keep pace with the need." The disease produced by the 1962 Oklahoma isolate was similar to that described by Bool (1959) who worked with a strain isolated in

the Netherlands Antilles.

Huxsoll, et al.,(1970a) recently reported a highly fatal syndrome termed tropical canine pancytopenia "...a newly recognized disease of dogs in diverse tropical and subtropical areas." Epistaxis was the most dramatic sign of this syndrome, and E. canis has been found consistently in affected dogs. Clinical and clinico-pathologic findings concerning tropical canine pancytopenia were reported by Walker, et al., (1970) and included severe anemia, leukopenia and thrombocytopenia in experimentally infected animals.

A relatively non-pathogenic strain of E. canis was reported from Arkansas by Ewing, et al., (1971). The organism in this case was found primarily in neutrophils rather than in lymphocytes and monocytes and produced a milder form of canine ehrlichiosis than the Oklahoma isolate found in 1962. A similarly less pathogenic neutrophilic strain of E. canis was isolated by the author in Oklahoma in 1970 and is compared in the present study with the 1962 Oklahoma isolate.

Although studies on the pathogenesis of canine ehrlichiosis caused by lymphocytic and monocytic isolates have been conducted by Ewing and Buckner (1965a) and Walker, et al.,(1970), certain aspects still remain unexplored.

It is the purpose of this investigation to re-examine the pathogenesis of the 1962 Oklahoma isolate of E. canis and to compare it with the newly found 1970 Oklahoma isolate. Throughout this paper the terms "1962 Oklahoma isolate" and "lymphocytic isolate" will be used interchangeably as will the terms "1970 Oklahoma isolate" and "neutrophilic isolate." In neither case are the white blood cells

named the only ones parasitized by that strain of E. canis. They are, however, by far the predominant cell type found to harbor morulae in the respective strains.

CHAPTER II

REVIEW OF LITERATURE

Causative Agent

Ehrlichia canis (order Rickettsiales) causes a febrile, sometimes debilitating, disease of dogs characterized by anemia. In most of the Old World literature canine ehrlichiosis is referred to as canine rickettsiosis because the causative organism was originally assigned to the genus *Rickettsia* (Ewing 1969).

Geographic Distribution

Donatien and Lestoquard (1935 and 1936a) working at the Pasteur Institute in Algeria, recorded for the first time a disease of dogs associated with appearance of inclusion bodies in monocytes of the peripheral blood. They later determined that these inclusions were rickettsiae and described a new species, *Rickettsia canis*. Donatien and Lestoquard (1935) stated that "...*Rickettsia canis* is not restricted to Algeria but also occurs along the Mediterranean Coast of France." The disease was later recognized in various parts of the Old World. Danks (1937) and Carmichael (1939) reported the occurrence of rickettsiosis (ehrlichiosis) from East Africa. Outbreaks in the Union of South Africa were characterized by large scale mysterious deaths among domestic and wild dogs in the Kruger National Park. These were reported by the park warden and game rangers and

investigated by Neitz and Thomas (1938) who conducted subinoculation experiments. These workers detected the presence of Rickettsia canis in the peripheral blood and organ smears and recognized it as a cause of disease. Lawrence (1938) was the first to recognize ehrlichiosis in South Rhodesia. He pointed out that the disease was confused with biliary fever (babesiosis) and therefore was rarely diagnosed correctly as being of rickettsial origin. A disease of dogs was also recognized and described in the French Congo in which rickettsial bodies resembling Rickettsia canis were found in monocytes of the peripheral blood, lungs, and liver (Malbrant 1939). He was uncertain whether this was the same organism as the South African one since he was able to infect guinea pigs and rabbits, findings contrary to those of other workers. Pigoury and Bernard (1939) reported inclusion bodies similar in morphology and staining characteristics to Rickettsia canis in monocytes of the lungs of a stray dog in Beirut. Alexander and Mason (1939) isolated the Rickettsia from peripheral blood of a dog in South Africa, and thus the disease was reported in various parts of Africa and the Orient within four years of its discovery.

Carmichael and Fiennes (1942) described a number of cases of the disease in Uganda and referred to it as "canine tick typhus." Gillain (1942) diagnosed rickettsiosis in association with Babesia canis infections among native breeds of dogs in the Belgian Congo. He suggested that "...Rickettsia canis does not cause a serious disease, but when associated with Babesia canis it may lead to a relapse to the latter organism." Mornet (1942) reported the presence of rickettsial infection among two dogs of European breeds in French West Africa. Mudaliar (1944) first reported the presence of

Rickettsia canis in South India; the organisms were found in monocytes and lymphocytes. This dog had persistent fever and died of the illness.

Malbrant (1945) reported another species of Rickettsia as the cause of conjunctivitis among dogs at Brazzaville in the French Congo. The organism was usually found in the epithelial cells of the conjunctiva and was named Rickettsia donatieni. The infection was thought to be contagious because several dogs were affected at the same time in the same area. Malherbe (1947) reported Rickettsia canis among dogs in Pretoria district of Africa where the infection was observed concurrently with Babesia canis infections. Girard and Rousselot (1947) recognized two forms of rickettsial disease in Sudan, a latent or Mediterranean type and an acute form in which ear shaking was a feature. Malherbe (1948) discussed diagnosis, symptoms and therapy of rickettsiosis (ehrlichiosis). He mentioned that dogs near Onderstepoort often were found to have rickettsiosis but that unless other blood parasites complicated the infection, they did not become anemic. Receveur and Hugaud (1949) gave a brief description of a fatal case of rickettsiosis in Chad. Abdussalam (1949), as cited by McGaughey, et al., (1962) stated that "...E. canis is the most pathogenic 'Rickettsia' of animals in India, Pakistan and that it has recently been found in W. Pakistan and S. India." He postulated that large numbers of cases diagnosed as "Lahore canine fever" in India and Pakistan were attributable to this infection. Occurrence of ehrlichiosis, then, was recognized in various parts of the Old World following its discovery in Algeria.

Reihart, et al., (1952) published a preliminary report of a dog

having "malignant canine lymphocytopenia" in Nebraska. They demonstrated inclusion bodies in the lymphocytes occupying 1/3-3/4 of the cytoplasm. It was stated that further studies were in progress but no further references have been found. The first confirmed report of the presence of E. canis in the New World was published by Bool and Sutmoller (1957) who detected E. canis in blood smears from four of seven dogs examined on the Island of Aruba (Netherlands Antilles). They successfully infected a dog in Utrecht by allowing Rhipicephalus sanguineus specimens taken from Aruban dogs to feed on it. Typical E. canis morulae were found in blood and organ smears of the experimental dog. They also found Babesia canis to be a complicating factor among naturally infected dogs as had workers in the Old World. At about the same time, the continued occurrence of Rickettsia canis (E. canis) in Africa and India was reported by Cassard (1957) and Raghavachari and Reddy (1958), respectively. The latter report indicated that ehrlichiosis was found only in pure-bred Alsatian and Golden Retriever dogs in Hyderabad, and the Indian strain of the organism was morphologically indistinguishable from African strains. McGaughey, et al., (1962) recorded the case histories of five dogs suffering from ehrlichiosis in Ceylon. Ewing (1963) reported the occurrence of leukocytic inclusion bodies in a Beagle pup with babesiosis in Oklahoma. In subinoculation experiments the inclusion bodies were consistently found in the cytoplasm of leukocytes including lymphocytes, monocytes, and neutrophils at a time just prior to appearance of large numbers of B. canis trophozoites in the peripheral blood. The nature of these inclusions and their relation to babesiosis was not determined initially. Later, however, the

inclusions were identified as Ehrlichia canis morulae (Ewing, 1964a and 1964b), and this was the first confirmed report of Ehrlichia canis in the United States.

Interest revived in Ehrlichia canis when Wilkins, et al., (1967) reported death of a large number of military dogs in Singapore due to a "new disease." The disease was first encountered late in 1963 but an acute flare up occurred from 1965-1967 when many dogs died. The etiologic agent of this disease was thought initially to be a virus. It was later recognized by Spence, et al., (1967) that this "new disease" was prevalent among a large number of native civilian dogs in addition to the military dogs, and it was considered to be most prevalent in the Alsatian breed.

In 1969 (Anonymous, 1969 and Weber, 1970) the occurrence of a condition termed "idiopathic hemorrhagic syndrome" was reported in United States military dogs in Vietnam. Huxsoll, et al., (1969) reported a "hemorrhagic disease" among military dogs in Southeast Asia and advanced the opinion that this was the same disease observed by British workers (Wilkins, et al., 1967 and Spence, et al., 1967) in military and civilian dogs in Singapore. A common aspect of this syndrome was severe epistaxis. Ehrlichia canis was suspected as the etiological agent of the disease since it was demonstrated consistently in the monocytes of affected dogs, and the term tropical canine pancytopenia (TCP) was adopted to refer to this syndrome in military dogs. Seamer and Snape (1970) observed a similar disease in dogs from the Persian Gulf region and conducted subinoculation experiments in the United Kingdom. E. canis was always found in the monocytes and lymphocytes of the experimental dogs, and they developed pancytopenia

and fever. Epistaxis was not characteristic, however, as it had been with TCP, and they suggested that some additional unrecognized factors might be involved in producing TCP. Walker, et al., (1970) recognized TCP in Vietnam, among several Labrador Retrievers which were shipped to Vietnam after having been trained in Malaysia as tracker dogs. They also reported an enzootic of TCP among military dogs of United States origin stationed in the Republic of Vietnam. One hundred and sixty fatal cases were recognized between 1968 and July 1969. They mentioned that "...the affected units are located throughout the Republic of Vietnam including highland, coastal plain and delta regions." Huxsoll, et al., (1970a and 1970b) reported Ehrlichia canis in Puerto Rico, Virgin Islands, and in Florida in addition to Southeast Asia. Ewing, et al., (1971) reported the presence of Ehrlichia canis in Arkansas. The organism they found was primarily in the neutrophils and produced a milder form of ehrlichiosis than the 1962 Oklahoma isolate which parasitized lymphocytes and monocytes.

Life Cycle and Transmission

The life cycle of E. canis (Rickettsia canis) has not been studied very extensively. Donatien and Lestoquard (1940) described the life cycle of Rickettsia canis (E. canis) in the cytoplasm of monocytes. The first form of Rickettsia canis (E. canis) recognized by these workers was a dark reddish homogeneous circular mass, the so-called "initial body." According to these workers the initial bodies underwent reproduction, fragmentation or both and developed into the mulberry or morula, the stage usually recognized as characteristic

of E. canis. These morulae are actually colonies of "elementary bodies" which separate or disintegrate into individual "elementary bodies" which enter other monocytes and develop into "initial bodies." A similar cycle was observed by Malbrant (1945) while studying canine conjunctivitis caused by Rickettsia donatieni. Bool (1959) in his studies of E. canis also demonstrated the presence of morulae and elementary bodies in the cytoplasm of monocytes, but he could not confirm the existence of an "initial body" in these cells. Ewing (1965 and 1969) found two kinds of intracytoplasmic inclusions in the cells of the agranulocytic series of dogs experimentally infected with Ehrlichia. "Those in lymphocytes are more apt than those in monocytes to be of the basophilic morula type, i.e., the type which is considered to be characteristic of Ehrlichia sp. Monocytes were sometimes observed to harbor morulae but not as commonly as were lymphocytes, and the inclusions which were characteristically found in monocytes were acidophilic and not made up of an aggregate of smaller units." He suggested that the monocytic inclusions might be identical to the initial bodies described by Donatien and Lestoquard (1940) but stated that "Conclusive evidence that they arise from elementary bodies was not found." Likewise, he had enough evidence to conclude that morulae disintegrate into elementary bodies, but direct evidence of a life cycle was not found. Small slate-gray inclusions were also observed consistently in the cytoplasm of neutrophils by Ewing (1963 and 1965) but the nature and significance of these inclusions was not determined.

In addition to the domestic dog as host of R. canis (E. canis), Donatien and Lestoquard (1935) found that monkeys (Macacus inuus)

could be infected experimentally and that a febrile reaction was produced. The typical morula stage of the organism was demonstrable in the peripheral blood. Opinion was advanced by Neitz and Thomas (1938) that wild dogs (Lycaon pictus) acted as reservoir hosts of E. canis in the Kruger National Park in Africa. Jackals (Thos mesomelas) were found to be susceptible to experimental infection but developed no symptoms although the parasites were demonstrable by subinoculation of blood into dogs after a period of 112 days. Malbrant (1939) described a disease of dogs in which he found a parasite morphologically identical to Rickettsia canis which was transmissible to guinea pigs and rabbits by intravenous inoculation, but monkeys were refractory. This finding was contrary to those of Donatien and Lestoquard (1935) and Neitz and Thomas (1938). Ewing, et al., (1964) demonstrated experimentally that the coyote (Canis latrans frustrar) could serve as a host for E. canis. They also were able to transfer the organism from infected coyotes back to susceptible dogs and concluded that coyotes were a potential reservoir host of E. canis.

Ticks have been incriminated as the vectors of Ehrlichia sp. Donatien and Lestoquard (1936a, 1936b, and 1937) showed that the brown dog tick, Rhipicephalus sanguineus, served as the vector of E. canis in Africa. Infection was found to be transmitted transovarially from the adult female to the next generation and all stages of the tick could be infective. These findings have been confirmed both circumstantially (Mudaliar, 1944; Raghavachari and Reddy, 1958; McGaughey, et al., 1962; Ewing and Buckner, 1965a; Huxsoll, et al., 1970a and 1970b; Nims, et al., 1971; and Walker, et al., 1970), and

experimentally (Bool, 1959). Some experimental attempts to transmit the organism through Rhipicephalus sanguineus to susceptible dogs have been unsuccessful; however, Rhipicephalus sanguineus recognized by the Old World workers as a transmitter of E. canis is now known to be a complex species and this may be a factor affecting the transmission studies (Ewing and Philip, 1966).

Infection could be transmitted experimentally by intravenous injection of infective whole blood, (Bool and Sutmöller, 1957; Raghavachari and Reddy, 1958; Bool, 1959; Ewing and Buckner, 1965a and 1965b; and Huxsoll, et al., 1970a and 1970b) and organ extracts (Bool, 1959 and Ewing and Philip, 1966). Whole blood transfusion thus could become a very important mechanism of accidental transmission of E. canis (Ewing, 1969). Recovered dogs act as reservoirs for extended periods of time since the organism can persist in convalescent animals for more than 29 months (Ewing and Buckner, 1965b). Jadin, et al., (1968, cited by Ewing 1969) suggested that the closely related "...Rickettsia prowazeki has a resistant stage which persists in the endothelial cells and that relapses occur when immunity is reduced." Ewing and Buckner (1965b) recognized the persistence of E. canis in convalescent dogs for long periods of time but conclusive evidence of reservoir sites could not be found.

Symptomatology

Although canine ehrlichiosis does occur as a distinct disease entity, concurrent infections of E. canis and other hematozoans are commonly recognized in natural outbreaks. Babesia canis has been mentioned very commonly as a companion with E. canis infections in

the Old World (Donatien and Lestoquard, 1937 and Lawrence, 1938) and in the New World (Bool and Sutmöller, 1957; Ewing, 1963 and 1964b; and Ewing and Buckner, 1965a). These concurrent infections usually result in a complex syndrome, and clinical findings are often confusing.

It is generally agreed that after an incubation period of one to three weeks (Malherbe, 1947 and 1948; Haig, 1955; Bool and Sutmöller, 1957; Raghavachari and Reddy, 1958; and Ewing and Buckner, 1965b), a rise in body temperature occurs, often the first symptom of the disease. Temperature curves are often very characteristic, showing exacerbations and remissions on alternate days reaching a peak of 106 to 107°F and then gradually coming down to lower peaks and to the normal range (Neitz and Thomas, 1938; Lawrence, 1938; Malherbe, 1947 and 1948; Haig, 1955; Bool and Sutmöller, 1957; and Ewing and Buckner, 1965a and 1965b). There is usually nasal and ocular discharge, the latter bilateral and often mucopurulent. The breath often is fetid (Neitz and Thomas, 1938; Raghavachari and Reddy, 1958; and Ewing and Buckner, 1965a). General symptoms, i.e., anorexia, emaciation, dehydration, vomiting, and diarrhea may also be seen (Ewing and Buckner, 1965a and Ewing, 1969). Superficial lymph nodes and the spleen are easily palpable (Bool and Sutmöller, 1957; McGaughey, et al., 1962; Ewing and Buckner, 1965a; and Ewing, 1969).

Malbrant (1939) described the disease in three forms, "...a nervous form characterized by convulsions, paralysis and symptoms of encephalomeningitis, a typhoid form characterized by fever, tachycardia, dyspnoea, and lymphadenitis, and a chronic form in which the symptoms may be variable." Carmichael and Fiennes (1942) in

Uganda also differentiated the disease into three forms: (1) cutaneous form characterized by absence of fever but presence of erythematopustular lesions in axillary and groin regions; (2) septicemic form, the most common manifestation of the disease, characterized by high fever and anemia; and (3) nervous form characterized by altered gait. Ehrlichiosis was found to occur essentially in the septicemic form by Malherbe (1947). He also found skin lesions in some instances of natural infection. He stated, however, that the different syndromes described by Carmichael and Fiennes (1942) tended to merge in any given animal and that their classification could not be regarded as rigid.

A series of papers concerned with a syndrome variously called "idiopathic hemorrhagic syndrome" and "tropical canine pancytopenia" has been published in recent years (Weber, 1970; Huxsoll, et al., 1969, 1970a and 1970b; Seamer and Snape, 1970; Walker, et al., 1970; and Nims, et al., 1971). Ehrlichia canis eventually was demonstrated in the peripheral blood of the infected dogs and is now considered to be the causative agent. Huxsoll, et al., (1969) and Seamer and Snape (1970) thought that unilateral or bilateral epistaxis was the initial phase of the disease while Walker, et al., (1970) later recognized that epistaxis and other hemorrhagic manifestations were terminal aspects of the syndrome. They divided the syndrome into three distinct phases: (1) a febrile phase characterized by sudden onset of fever and general signs, i.e., anorexia, decreased stamina, and weight loss; (2) a subclinical phase following the febrile period, during which most of the dogs regained the weight lost earlier, looked healthy, but laboratory studies showed altered hemograms; and (3) a terminal

phase which was subdivided into (a) severe pancytopenia but no bleeding, and (b) epistaxis. Death often occurred in this latter phase in from 1 to 5 days.

As mentioned elsewhere, an Ehrlichia sp. was recognized recently in horses by workers in California. Stannard, et al., (1969) and Gribble (1969) studied equine ehrlichiosis and found a rise in body temperature up to 107°F two days after intravenous exposure to infective blood. Anorexia, depression, edema of the legs and ataxia were commonly seen in the infected horses. Gribble (1969) stated that "...because the clinical signs in horses with equine ehrlichiosis are often so striking and yet the incidence of the disease is low, at least as indicated by the relatively few cases we have seen, it may be that the horses represent an abnormal host." It has also been determined that the agent isolated from horses is transmissible to other animals, including sheep, goats and dogs, but the clinical signs attendant with infection in these species were either mild or non-existant.

Hematology and Chemistry

There are many reports in the literature describing concurrent E. canis and Babesia canis infection (Donatien and Lestoquard, 1937; Lawrence, 1938; Neitz and Thomas, 1938; Bool and Sutmoller, 1957; and Ewing, 1963, 1964a and 1964b). Ewing (1969) stated that "...uncomplicated ehrlichiosis is probably a relatively mild disease except in young dogs, or in debilitated older ones. Furthermore, hematozoan diseases and such ill-defined syndromes as the distemper complex in dogs are so widespread as to increase the probability that

E. canis may occur in concert with other pathogens." Shirlaw (1938, cited by Ewing 1969) described a disease known as "Lahore canine fever" which was unquestionably a result of mixed infection of Babesia canis and E. canis. He observed aggregates of granules morphologically similar to morulae in the cytoplasm of polyblasts and reticular cells.

Donatien and Lestoquard (1935 and 1936b) and Neitz and Thomas (1938) observed that dogs suffering from rickettsiosis (ehrlichiosis) were anemic. Lawrence (1938) found evidence of anemia, anisocytosis and well-marked polychromasia. Malherbe (1948), on the other hand, saw no sign of anemia in uncomplicated cases. Carmichael and Fiennes (1942) observed an enormous increase in monocytes about the 13th day of infection and most monocytes resembled lymphocytes with a simple round nucleus. Eosinophils disappeared completely. Monocytosis was also observed by Mudalier (1944) and Haig (1955).

Reihart, et al., (1952) gave a brief description of a "new disease" in dogs characterized by "...an absolute lymphopenia ranging from 0.5 percent to 17 percent. The total white cell count varied from 2,400 to 47,000 per cu. mm. The leukopenia was often present early in the disease." Unidentified intracytoplasmic inclusions were seen in lymphocytes. These workers stated that further studies were in progress, but apparently no further report was published. Bool and Sutmöller (1957) observed severe anemia characterized by anisocytosis, polychromasia, poor blood coagulation, and fast sedimentation rate in canine ehrlichiosis. They also found an early monocytosis and eosinopenia.

Raghavachari and Reddy (1958) observed changes characteristic of

anemia in blood smears of animals having uncomplicated E. canis infection and reported a considerable increase in mononuclear leukocytes.

The first detailed study on hematologic changes caused by ehrlichiosis was conducted by Bool (1959) who reported hypochromic anemia in experimentally infected dogs. During the period from the first days of fever, and persisting for 3 to 4 months, normoblasts were always present as were polychromasia and anisocytosis. There was a sharp decline in hemoglobin and in red blood cells. Sedimentation rate was increased. Total number of leukocytes decreased soon after exposure and then rose in the first phase of fever. Monocytosis was not observed in their studies. McGaughey, et al., (1962) observed monocytosis and eosinopenia. A series of experiments on babesiosis, ehrlichiosis and combined infection in dogs conducted by Ewing and Buckner (1965a) showed that "a grave illness accompanied by severe anemia of normocytic-normochromic type, developed in dogs concurrently infected by Babesia canis and Ehrlichia canis. Hematologically it was found that anemia was twofold; i.e., destruction of mature erythrocytes and impediment of erythropoiesis. Babesia appeared to be responsible for the former and Ehrlichia for the latter. Dogs affected by either parasite in the absence of the other did not succumb as often. Nevertheless, anemia was long standing in dogs with ehrlichiosis infections, and young dogs often died. Dogs with Babesia infections developed anemia but, through active hemopoiesis, recovered from it."

The term tropical canine pancytopenia was proposed by Walker, et al., (1970) to describe the Ehrlichia-induced hemorrhagic syndrome

occurring in military dogs in Southeast Asia. They observed normocytic anemia during all phases of the disease. Normal bilirubin values ranging from 0.1 to 1 showed that anemia was of a non-hemolytic type. Blood urea nitrogen levels were significantly elevated at the terminal phase of TCP. Alkaline phosphatase and serum glutamic oxalacetic transaminase were slightly above normal limits but were not significantly different from control animals. They also stated that "...the biochemical or physiologic causes of bleeding in TCP are not yet known. Prothrombin time and clotting time were always normal, even though bleeding time exceeded 30 minutes."

Burghen, et al., (1971) in their serum electrophoretic studies of infected dogs found significantly increased gamma globulin concentrations and decreased serum albumin concentration.

The effect of Ehrlichia canis on platelets has not been studied in detail. A few brief accounts are, however, available in the literature. Carmichael and Fiennes (1942) and McGaughey, et al., (1962) observed an increase in the number of platelets in early stages of canine rickettsiosis, and these platelets were found to be parasitized by Rickettsia canis (E. canis). Bobin, et al., (1962) observed a severe thrombocytopenia in dogs suffering from a syndrome in which the main sign was epistaxis. Wilkins, et al., (1967) reported severe depression of all cellular components of the blood, including thrombocytes, in a disease syndrome now recognized to have been ehrlichiosis in dogs from Singapore. A marked but transient thrombocytopenia was observed by Foster and Cameron (1968) in sheep suffering from tick-borne fever, a rickettsiosis. Ewing (1969), in his well-documented review paper, mentioned that clotting time is

increased in canine ehrlichiosis, but he was uncertain whether this was related to thrombocytopenia. A decrease in thrombocytes was also observed in equine ehrlichiosis by Gribble (1969) who mentioned that "...thrombocytopenia usually occurred on days 4 through 12 of the disease when less than 50,000 thrombocytes/cmm were a frequent observation. When the thrombocytes were counted daily, the onset of thrombocytopenia was usually seen to precede the onset of edema by approximately a day. Regression of edema usually preceded the return to normal number of thrombocytes." Huxsoll, et al., (1970b) found severe thrombocytopenia in dogs suffering from TCP.

Ehrlichia canis (Neutrophilic Isolate)

Reports of the occurrence of Ehrlichia canis primarily in the cytoplasm of neutrophils are rare. Neitz and Thomas (1938) found E. canis morulae in neutrophils occasionally but most were in monocytes. Inclusion bodies were described in the circulatory neutrophils of dogs affected with canine distemper (Cello, et al., 1959) but these were later shown, by means of fluorescent antibody studies, to contain a specific viral antigen. Ewing (1963, 1964b and 1965) and Ewing and Buckner (1965a and 1965b) consistently observed small slate-gray intracytoplasmic inclusions in the neutrophils of dogs experimentally infected with E. canis. These inclusions were observed early in the course of illness before the appearance of typical E. canis morulae in lymphocytes and monocytes. The nature and significance of these inclusions was not known. Typical morulae were also observed in neutrophils on some occasions in infected dogs, but they were of rare occurrence.

Schalm (1965) reported the occurrence of intracytoplasmic inclusions in the neutrophils of two horses in California. Leukopenia of varying degrees was noticed in these cases. It was concluded that these inclusions bore a morphologic resemblance to those of tick-borne fever of sheep (Foggie, 1951). Occurrence of neutrophilic inclusions, presumably Ehrlichia sp., in California horses was later confirmed by Stannard, et al., (1969) who conducted subinoculation experiments in two horses and found that eosinophils, as well as neutrophils, contained inclusions. He stated that "...following an incubation period of two days, the body temperature rose to 104°F and 105°F respectively, and the following day typical cytoplasmic inclusions were seen in the neutrophils of both horses." Normochromic, normocytic anemia and leukopenia were observed in all infected horses. Neutropenia and lymphopenia were also seen consistently. These authors tentatively suggested equine ehrlichiosis as a name for this disease. Detailed studies on the etiology, symptomatology and hematology were later conducted by Gribble (1969). Thrombocytopenia and decreased packed cell volume were consistently observed. They also noticed neutropenia and lymphopenia; neutrophils decreased more slowly than the lymphocytes.

Ewing, et al., (1971) observed the presence of Ehrlichia canis in Arkansas. They also found an apparent strain difference between this isolate and the one found in Oklahoma in 1962. In dogs affected by the Arkansas isolate, morulae were found primarily in neutrophils rather than in lymphocytes and monocytes and produced an even milder form of canine ehrlichiosis than the Oklahoma isolate found in 1962. In their subinoculation studies in two dogs, a temperature rise

occurrred in both dogs on the 16th day and typical morulae were found in peripheral blood neutrophils on the 20th and 21st days post exposure and persisted for 6 to 7 days. Another dog was sub-inoculated and the organism was recovered predominantly in the neutrophils. This was the first report of the occurrence of this mild strain of E. canis. Detail studies on hematology and chemistry were not reported.

CHAPTER III

MATERIALS AND METHODS

Infectious Agents

The lymphocytic strain of Ehrlichia canis (1962 Oklahoma isolate) was obtained from a Beagle dog maintained as a reservoir of this organism by the Department of Veterinary Parasitology and Public Health, Oklahoma State University.

The neutrophilic strain of Ehrlichia canis (1970 Oklahoma isolate) was obtained from a female German Shepherd which was admitted to the Small Animal Clinic at Oklahoma State University on July 2, 1970, and later determined to have ehrlichiosis. Ten ml. of blood were obtained from this dog and 5 ml. injected intravenously into each of two pups. Both pups experienced slight pyrexia, and E. canis morulae were observed in leukocytes, primarily neutrophils and occasionally eosinophils. One of these two pups was kept as a reservoir and was the source of blood used in the experiments described in this thesis.

Experimental Animals

Three litters of pups were used in this study. Litter number one, composed of ten pups of mixed breeding (German Shepherd and Collie), was 6 weeks of age when pre-exposure observations were begun. Litters number two and three were composed of 6 and 7 pups, respectively and were also mongrels; the pups for the second experiment were a mixture

of Chow and Collie while the pups for the third experiment were English Setter and Collie. Like litter one, they were approximately 6 weeks of age when pre-exposure observations were begun.

All pups were housed in clean quarters which excluded other animals except arthropods. Ticks were never found on any of the pups or in the room; however, a few cockroaches of the family Blattidae and house flies (Musca domestica) were observed in the room occasionally. In each experiment, littermate controls were kept in similar cages adjacent to the infected dogs throughout the study. Ample bedding of newspapers was provided and changed twice a day. The cages were scrubbed, washed and disinfected at least once a day. The dogs were provided with fresh food[1] and water twice a day throughout the studies. They were vaccinated[2] to protect against canine distemper and infectious hepatitis. Fecal samples were examined for parasite eggs and larvae by direct saline smear and by sodium nitrate flotation method as described by Bello (1961). All pups used in the second and third experiments were found to be infected with Ancylostoma caninum and Toxocara canis. They were treated for ancylostomiasis by subcutaneous injection of dinitrophenol[3] and for Toxocara infection by oral administration of piperazine citrate.[4] Litter number two also had Giardia infection and was treated with

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1 Purina Dog Chow; Ralston Purina Company; St. Louis, Missouri

2 Cabvac; American Cyanamid Company; Princeton, New Jersey

3 DNP; American Cyanamid Company; Princeton, New Jersey

4 Parlamate; Ormont Drug and Chemical Co., Inc.; Englewood, New Jersey

quinacrine hydrochloride[5] administered per os. Fecal samples were rechecked after treatments to make certain that dogs were free of parasitic infection. Blood smears from pups in all three litters were stained with Wright's stain (buffer 6.8) and examined for the presence of blood parasites. All the pups were found to be free of helminths and of blood parasites before exposure.

Bleeding Procedures and Handling of the Blood

Bleeding from Jugular Vein. Blood samples were drawn aseptically from the jugular vein with a syringe and either a 20 or 21 guage needle. Hair was removed from the area over the jugular vein and kept free by clipping with a small animal electric clipper (blade size 40). The pups were held in standing position by an assistant and the venipuncture site sponged with 70 percent alcohol. The vein was located by gentle pressure, the needle inserted into it and a quantity of blood drawn according to need. Five ml. of blood usually were collected; but 7.5 ml. were obtained once a week when prothrombin time was determined in addition to hemogram and the liver and kidney function tests. Blood collected in this manner was immediately transferred to appropriate tubes by the method as follows:

1. Two ml. of blood were placed in vacutainer tubes[6] containing 0.04 ml. of 15 percent solution of disodium salt of ethylene diaminetetracetic acid (EDTA) as the anticoagulant. The blood was mixed with the anticoagulant by gently inverting the tubes a few times.

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[5] Atabrine; Winthrop Chemical Company, Inc.; New York, New York

[6] Vacutainer; Becton-Dickinson and Company; Rutherford, New Jersey

This blood was later used to make the following determinations: complete hemogram (hematocrit, hemoglobin, total erythrocytes, reticulocytes, total leukocytes and differential leukocytes), blood urea nitrogen (BUN) and creatinine.

2. One ml. of blood for thrombocyte counts was placed in sterile silicone-coated tubes immersed in an ice-water bath maintained at approximately 0°C. Dilutions of whole blood with 1% ammonium oxalate solutions were made directly from the tube by using either red blood cell (RBC) or white blood cell (WBC) diluting pipettes. WBC pipettes were used to make 1:20 dilutions during periods of severe thrombocytopenia and RBC pipettes were employed for 1:100 dilutions when thrombocyte values were not so depressed. The procedure employed was that described in detail by Brecher and Cronkite (1964).

3. Two ml. of blood were placed in sterile tubes without anticoagulant and allowed to clot. After clot retraction at room temperature, serum was collected and used to determine glutamic pyruvic transaminase (SGP-T), serum glutamic oxalacetic transaminase (SGO-T) and total and direct bilirubin.

4. Two and a quarter ml. of blood were placed in a centrifuge tube containing 1/4 ml. of sodium oxalate and used to determine prothrombin time.

Bleeding from Capillary Bed of the Ear. On those days when pups were not bled from the jugular vein for hematological and chemical studies, smears were prepared from capillary blood of the pinna. These smears were used to determine parasitemia. Malherbe (1947 and 1948) recommended that a very shallow incision be made on the edge of the ear and that a small portion of the first drop of

blood to emerge be taken for determining parasitemia. With this method large numbers of leukocytes could be examined for the presence of E. canis morulae with a minimum amount of labor. Ewing (1963 and 1964b) and Ewing and Buckner (1965a) used similar methods in studies on E. canis. In the present investigation the principle of the above procedure was followed but slight modifications were made in methodology. The ventral surface of the pinna was shaved and cleaned with 70% alcohol and the capillary bed on this area was punctured with a sterile disposable lancet.[7] The first drop of blood which oozed from the puncture was drawn by capillary pressure into a coagulant-free capillary tube. The blood was then discharged onto a coverslip and smears were prepared. The detail of the method for preparing coverslip smears is described by Coles(1967).

Clinical Signs

A week before exposure to infective blood, the animals were subjected to thorough physical examination and determined to be in good condition. The body temperature was recorded daily, and blood and fecal samples were examined before the start of experiment. The pups were thoroughly checked for the presence of external parasites, especially ticks. After exposure both the principals and controls were examined each day for any sign of illness. Rectal temperatures were recorded at least once a day. Results of blood and fecal examinations were recorded throughout the prepatent, patent and post-patent infection periods.

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[7] Microlance; Becton, Dickinson and Company; Rutherford, New Jersey

Hematological Studies

Blood samples were obtained from all principals and the unexposed controls at regular intervals; specific schedules are described elsewhere in detail of individual experiments. Hematological methods described by Schalm (1965) were employed to ascertain the following:

1. Total red blood cell count
2. Total white blood cell count
3. Differential leukocyte count
    a. lymphocytes
    b. monocytes
    c. neutrophils
    d. eosinophils
    e. basophils
4. Reticulocyte count
5. Packed cell volume
6. Hemoglobin
7. Thrombocyte count

Total red blood cell and total white blood cell determinations were made by using certified pipettes for dilutions and the improved "Bright Line" Neubauer hemocytometer for the counting. For differential leukocytic counts, blood films were prepared on coverslips by the method described by Coles (1967). The blood films prepared both for differential leukocytic counts and for determination of parasitemia were stained with Wright's stain (buffer 6.8). Differential leukocyte counts were calculated in percentages from a total of 200 white blood cells. Absolute values were, however, used for inter-

pretation of results. Smears stained by new methylene blue and counter-stained by Wright's stain (buffer 6.8) were used to demonstrate the reticulocytes. The absolute number of reticulocytes among 1,000 erythrocytes was determined and recorded as a percentage. Plain capillary tubes[8] were used to determine packed cell volume. Cyanmethemoglobin[9] method was employed for hemoglobin determination.

The procedure described by Brecher and Cronkite (1964) was used for thrombocyte counts. Platelets were enumerated by using a flat-bottomed counting chamber and phase contrast microscopy. As explained earlier, dilutions of either 1:20 or 1:100 were made, according to the anticipated number of platelets present, with 1% ammonium oxalate. The shape and size of the thrombocyte was also observed.

Thromboplastin extract[10] was used to determine the prothrombin time. The method is described by the manufacturer in a bulletin enclosed with the chemical.

Chemical Studies

Serum glutamic oxalacetic transaminase (SGO-T) and serum glutamic pyruvic transaminase (SGP-T) values were determined by a colorimetric procedure described in a technical bulletin[11] published by Sigma Chemical Company.

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[8] Yankee Micro-Hematocrit; Clay-Adams, Inc.; New York, New York

[9] Hycel, Inc.; P.O. Box 36329; Houston, Texas

[10] Simplastin; General Diagnostic Division; Warner Chilcott Laboratories; Morris Plain, New Jersey

[11] Sigma Technical Bulletin Number 505; Sigma Chemical Company; St. Louis, Missouri

The diazo reaction method described by Malloy and Evelyn (1937) and modified by Henry (1965) was employed to determine the total and direct bilirubin levels in the serum.

The urease and the Berthelot method described by Henry (1965) was followed to determine blood urea nitrogen (BUN).

The method described by Owen, et al., (1954) and modified by Henry (1965) was used to determine creatinine levels in serum.

Quantitative Estimation of *Ehrlichia canis*

The progress of parasitemia was determined by the method used by Ewing (1964b) with a slight modification. Smears were made from capillary blood of the pinna on days when pups were not bled from the jugular vein for the battery of hematological and chemical studies. On days when blood was obtained from the jugular vein, a drop was taken from the sample and a coverslip smear prepared. Two thousand oil immersion fields were examined to determine parasitemia on Romanowsky stained (Wright's stain, buffer 6.8) smears of blood. Examination of blood was continued until termination of the experiment or death of pups whichever occurred first.

Detail of Experiments

Experiment 1. *Ehrlichia canis*, 1962 Oklahoma isolate. The details of pre-exposure studies have been mentioned earlier. Briefly, these included complete hemograms (hematocrit, hemoglobin, total red blood cells, reticulocytes and total and differential leukocytes), liver function tests [serum glutamic pyruvic transaminase (SGP-T) and serum glutamic oxalacetic transaminase (SGO-T) and total and direct bilirubin], renal function tests [blood urea nitrogen (BUN) and

creatinine], prothrombin time and thrombocytic studies (size, shape and number of thrombocytes).

Six of the ten littermate pups were exposed to the 1962 Oklahoma isolate of Ehrlichia canis by injecting each with 5 ml. of whole blood intravenously. Heparinized syringes and needles were used for this purpose. Four pups served as uninoculated controls. The ten pups were divided into two working groups, each composed of three exposed (principals) and two unexposed pups. Each group of pups was bled from the jugular vein twice a week, on alternate days, for the first two weeks and then three times a week. The thrice weekly schedule was followed until recovery of some of the pups, or alternatively, until all had died. On days when blood was not collected from the jugular, smears were prepared as described previously from capillary blood of the pinna, stained and examined to follow parasitemia. Rectal temperature and clinical signs were recorded daily. Postexposure studies were identical to those conducted before exposure to infectious blood. At the termination of the experiment control animals were challenged with Ehrlichia canis by intravenous inoculation of 5 ml. of blood from the same reservoir source.

Experiment 2. Ehrlichia canis, 1970 Oklahoma Isolate. This experiment was based on the same methodology as described for Experiment 1, but a litter of only 6 pups was used. After conducting pre-exposure studies described elsewhere, four pups (principals) were exposed to the neutrophilic isolate (1970 Oklahoma) of Ehrlichia canis, and the remaining two served as unexposed controls. Control pups were challenged at the end of the study.

Experiment 3. Both Isolates, Separately, in Littermate Pups.

Since the pups selected for Experiments 1 and 2 belonged to two different litters, a third experiment was conducted to study both isolates simultaneously. A litter of 7 pups was used; three pups were subjected to the lymphocytic isolate, an equal number to the neutrophilic isolate, and one pup served as an uninoculated control All studies were the same as those conducted for the first two experiments.

CHAPTER IV

RESULTS AND DISCUSSION

The results of the three experiments will be considered separately and discussion of these results will be interrelated. In addition, the results of this study will be integrated with findings of other authors.

Experiment 1. Ehrlichia canis, 1962 Oklahoma Isolate

Incubation Period and Parasitemia

Donatien and Lestoquard (1937) stated that the incubation period of canine ehrlichiosis varies from 6 to 12 days in natural cases and from 3 to 15 days in experimental cases. In most of the earlier published reports (Malherbe, 1947 and 1948; Haig, 1955; Bool and Sutmöller, 1957; Raghavachari and Reddy, 1958; Ewing, 1964b; and Ewing and Buckner, 1965b) the incubation period is said to range from 7 to 21 days. Ewing (1964b) stated that "...the incubation period of Ehrlichia can be judged by the occurrence of a febrile response or by the observation of intracytoplasmic inclusion in the leukocytes." He observed the appearance of lymphocytic morulae of E. canis (1962 Oklahoma isolate) in 12 dogs as early as 9 days postexposure to as late as 27 days postexposure; the average was 20.08 days. He reported a body temperature of at least 104°F to occur on the average of 11.33 days after exposure, with the range of 9 to 14 days.

In the first experiment of this study, 6 pups (#70,72,74,76,77 and 79) were experimentally infected with the 1962 Oklahoma isolate of E. canis, and 4 littermate pups (#69,71,78 and 81) were kept as uninfected controls. The results in this experiment are presented in Table 1 and in Figures 3,6,9,12,15,18,21,26 and 29. The lines on the graphs represent moving three-point averages of the mean values of principal and of control animals. In the present study the same 1962 Oklahoma isolate of E. canis used by Ewing in his experiments was utilized and appearance of lymphocytic inclusions in the form of typical morulae (Figure 1) were taken as evidence of infection. Typical morulae of E. canis were observed in all 6 infected pups as early as 11 days and as late as 16, the average being 13.67 days (Table I). These results agree closely with findings of Ewing (1964b).

The morulae persisted in peripheral blood lymphocytes (Table 1) for a period ranging from 44 to 54 days after the exposure. Large homogeneous inclusions (Figure 2) resembling the so-called initial body of Donatien and Lestoquard (1940) were also observed on a few occasions in monocytes. It cannot be stated definitely what stage of the life cycle these inclusions represent. Ewing (1969) mentioned that "Larger homogeneous bodies which might be mistaken for initial bodies are sometimes referred to as plaques." Coles (1953) and Haig (1955) believed that these plaques were really morulae in which the individual granules had been obscured by intense staining. In this study typical morulae were never found in either monocytes or neutrophils. Neitz and Thomas (1938) occasionally found morulae in the neutrophils of African dogs. Ewing (1964b) observed that lymphocytes were commonly affected but that monocytes and rarely neutrophils also harbored morulae.

TABLE I

INCUBATION PERIOD AND PERSISTENCE OF EHRLICHIA CANIS (1962 OKLAHOMA ISOLATE) IN PERIPHERAL BLOOD OF SIX PRINCIPALS ON DAYS AFTER EXPOSURE TO INFECTIOUS BLOOD

Identification No. of Pups	Incubation Period in Days	Persistence of E. canis Morulae on Days Post Exposure
70	16	16,17,18,19,20,21,22,23,24,26, 27,30,31,33,35,38,39,40,42,43, 44,46
72	11	11,12,13,14,16,17,18,19,21,22, 23,26,28,29,30,32,33,34,36,37, 38,39,41
74	13	13,14,15,16,17,18,19,20,21,22, 24,25,26,28,30,31,32,33,35,37, 39,40,44,46
76	15	15,16,17,18,19,20,21,23,24,25, 27,29,30,32,33,34,36,37,38,41, 42,43,47,49,50,52
77	13	13,14,15,16,17,18,19,20,21,22, 23,25,27,29,30,32,34,36,38,39, 40,43,44,46,47,48,49
79	14	14,15,16,17,18,19,20,22,24,25, 26,27,28,29,31,32,33,35,36,37, 38,40,41,42,44,45,46,47,49,52, 54

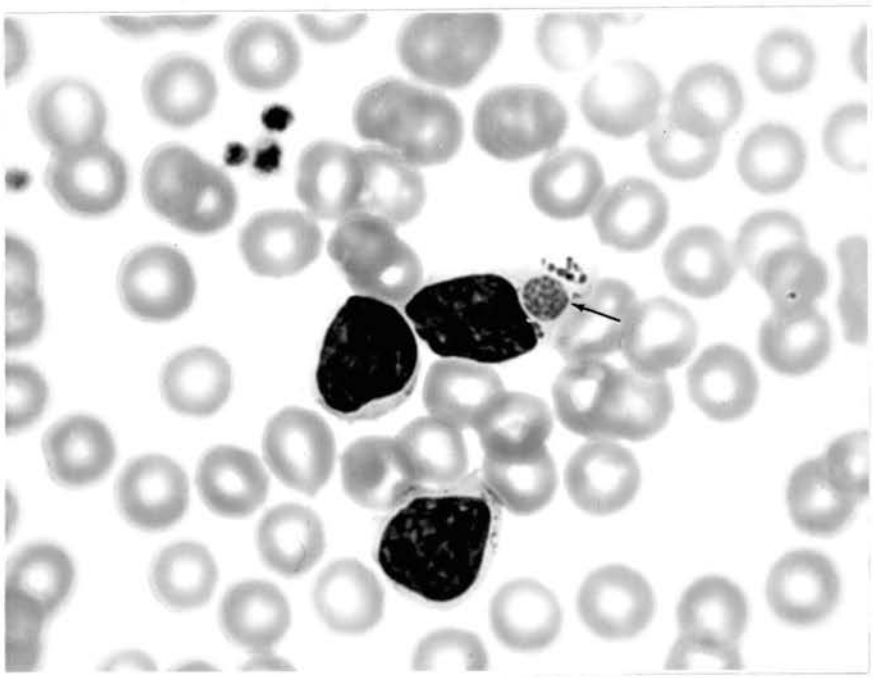

Figure 1. Lymphocyte containing morula of Ehrlichia canis, 1962 Oklahoma isolate

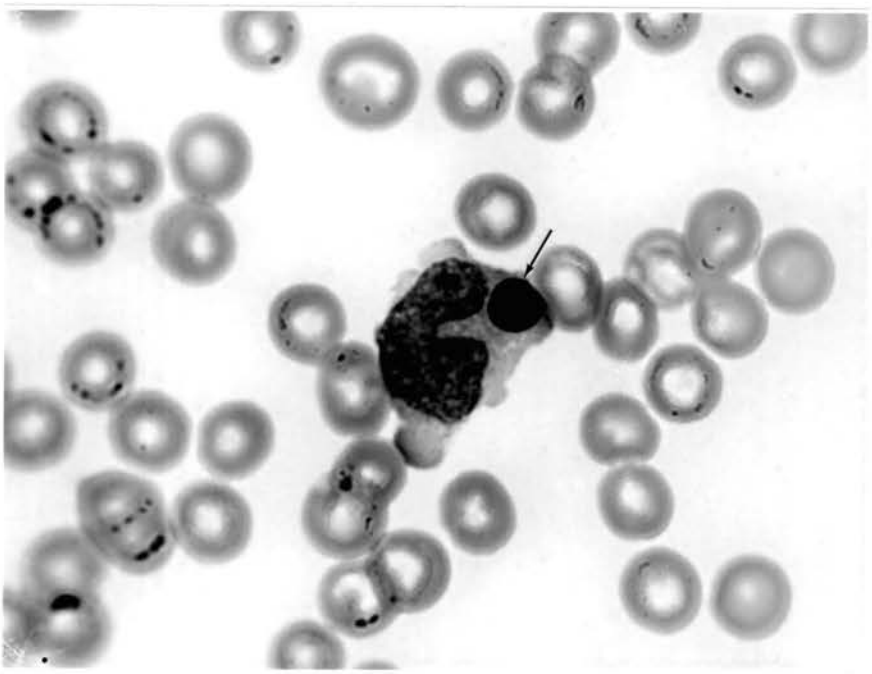

Figure 2. Monocyte containing a large homogeneous inclusion comparable to Ehrlichia canis, 1962 Oklahoma isolate, initial body described by Donatien and Lestoquard, 1940

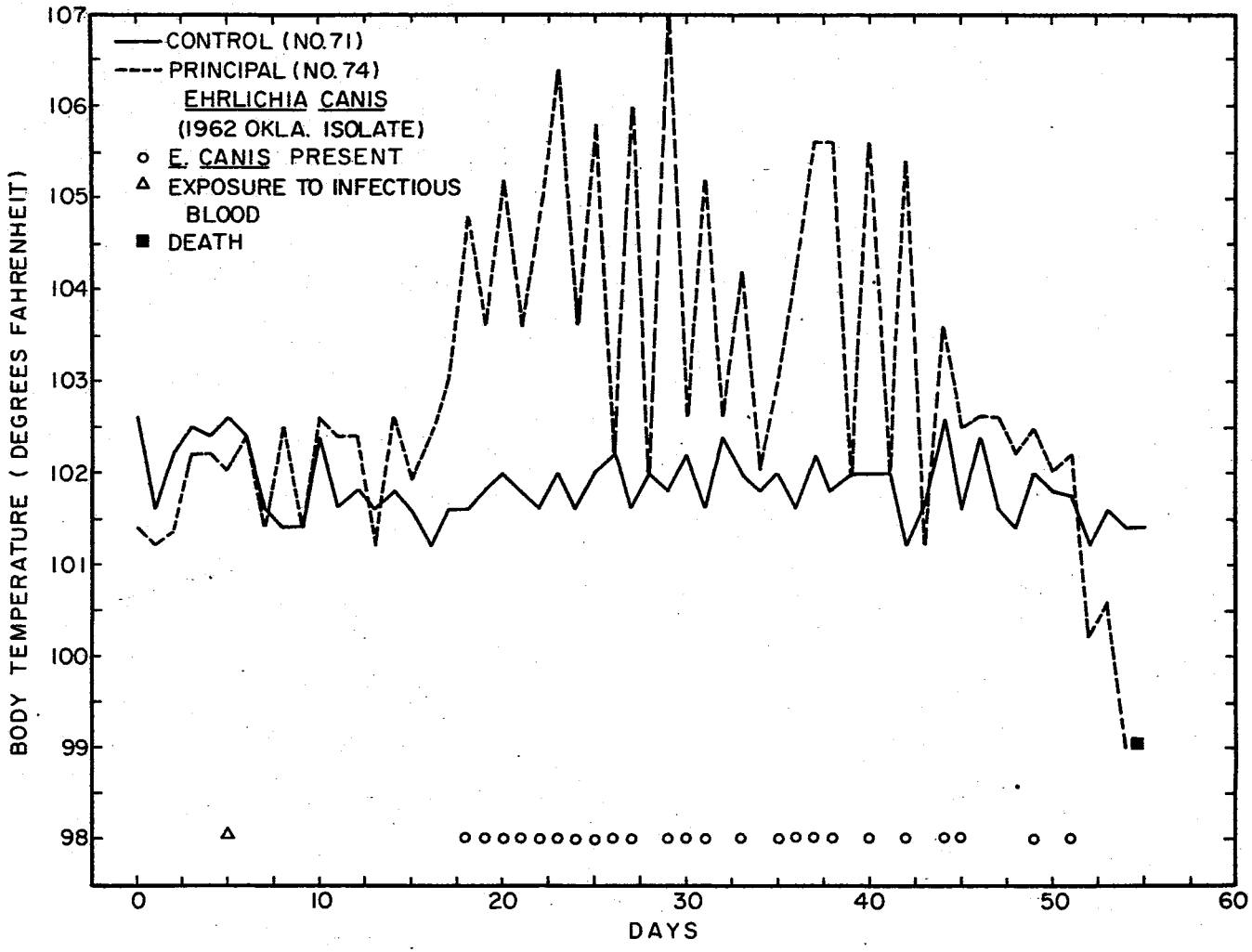

Figure 3. Parasitemia and body temperature fluctuations in a representative principal and a littermate control; principal exposed to E. canis, 1962 Oklahoma isolate.

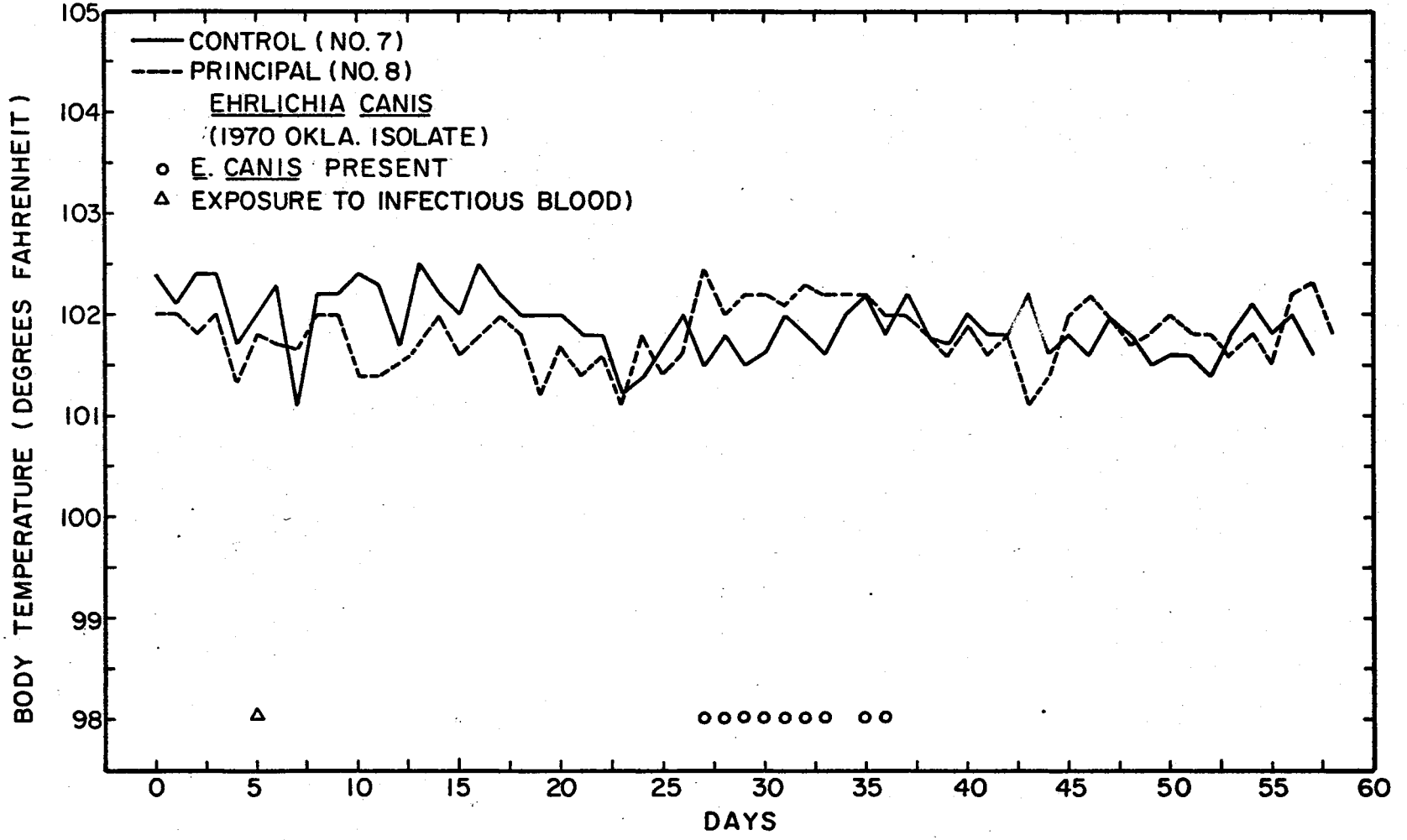

Figure 4. Parasitemia and body temperature fluctuations in a representative principal and a littermate control; principal exposed to E. canis, 1970 Oklahoma isolate

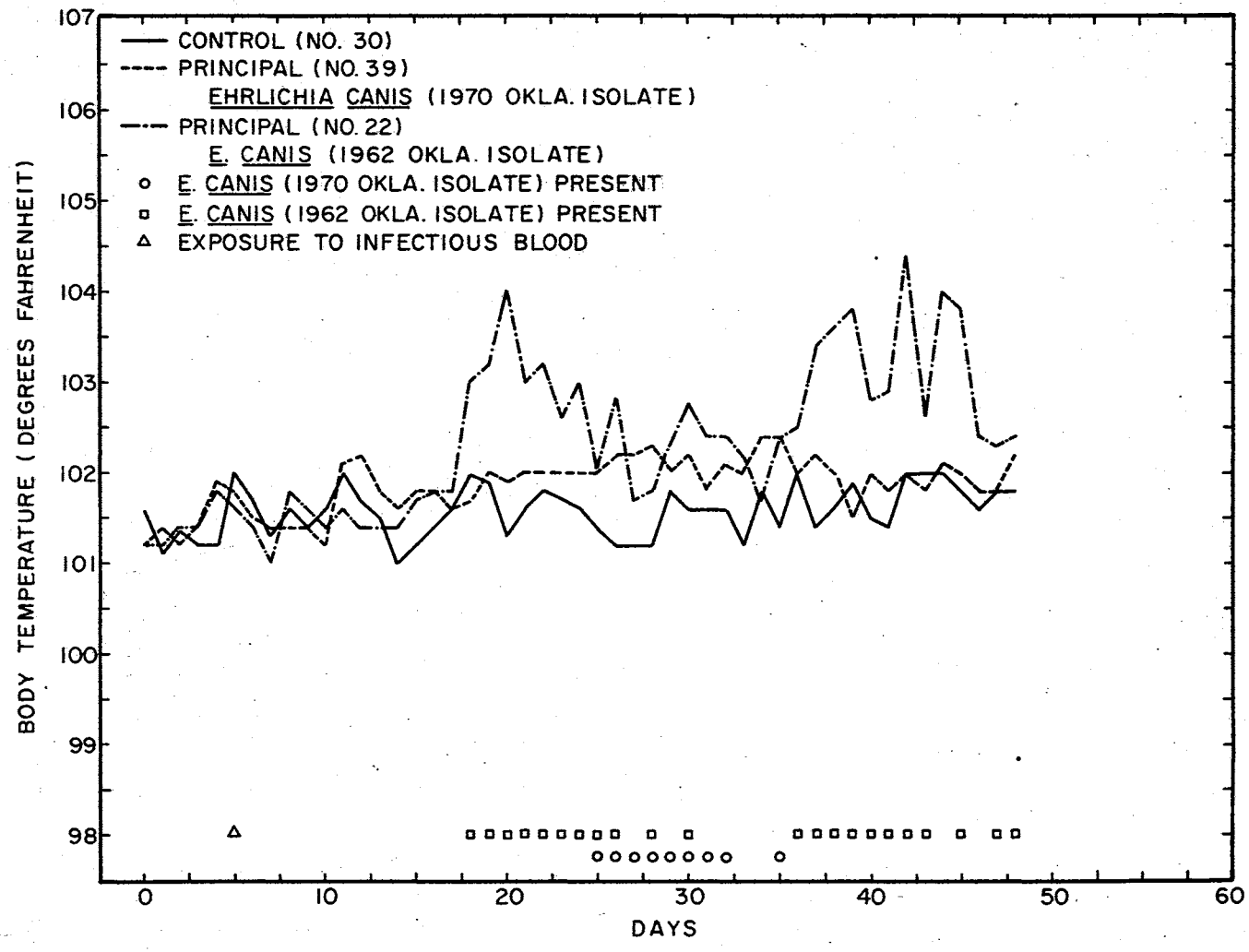

Figure 5. Parasitemia and body temperature fluctuations in two representative principals and a littermate control; one principal exposed to E. canis, 1962 Oklahoma isolate and the other to E. canis, 1970 Oklahoma isolate

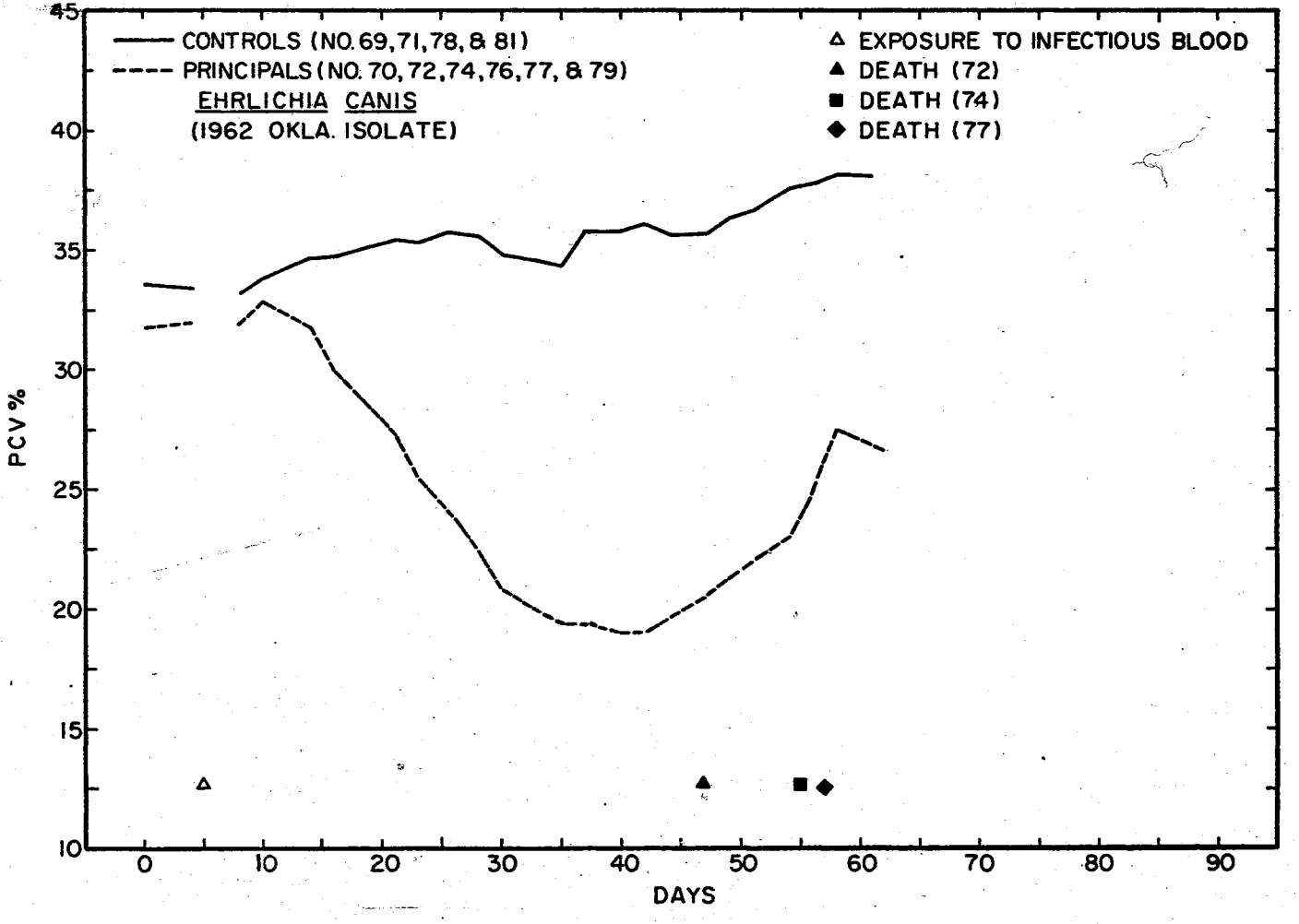

Figure 6. Moving averages of the mean packed cell volume of six principals and four controls; principals exposed to E. canis, 1962 Oklahoma isolate

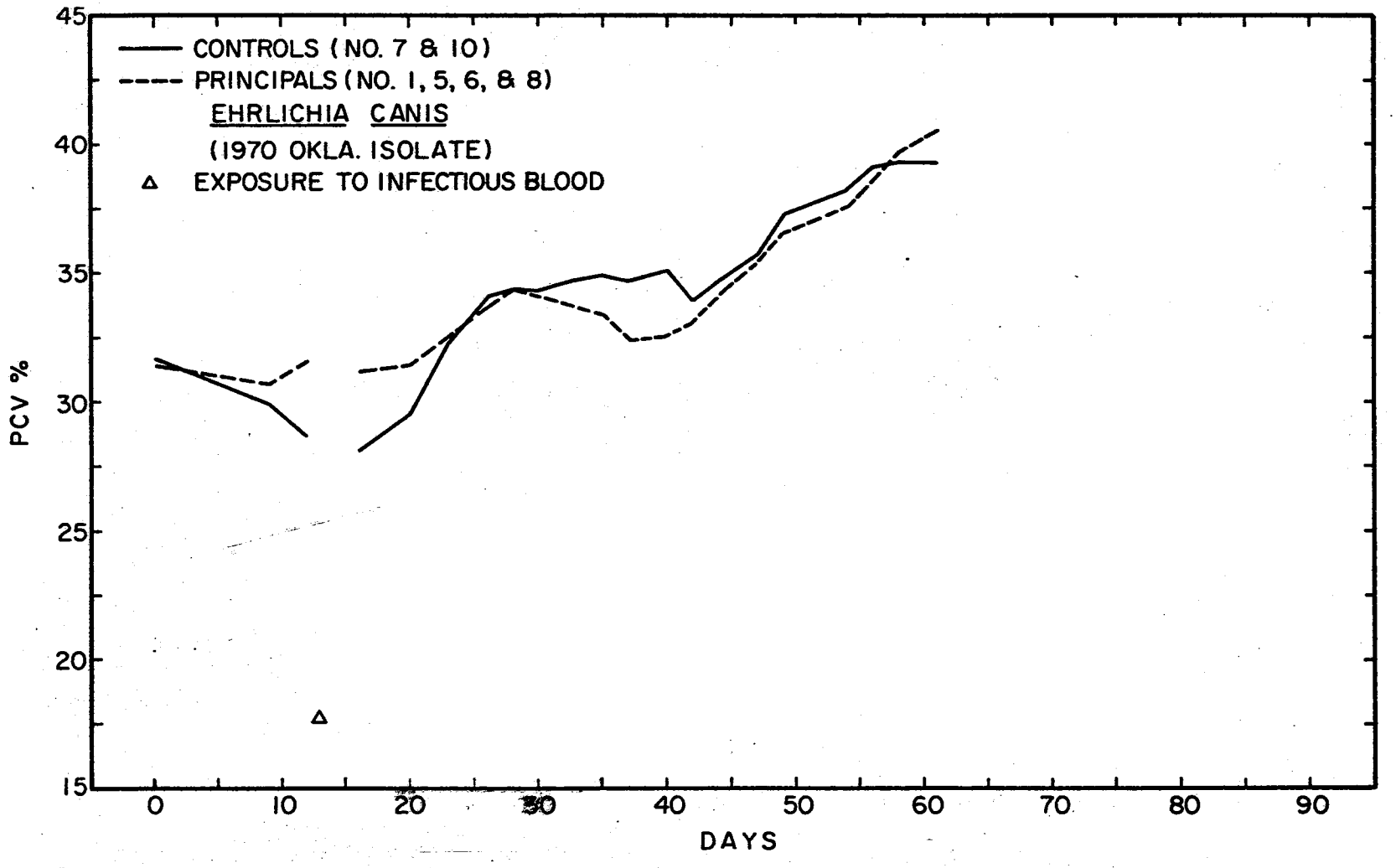

Figure 7. Moving averages of the mean packed cell volume of four principals and two controls; principals exposed to E. canis, 1970 Oklahoma isolate

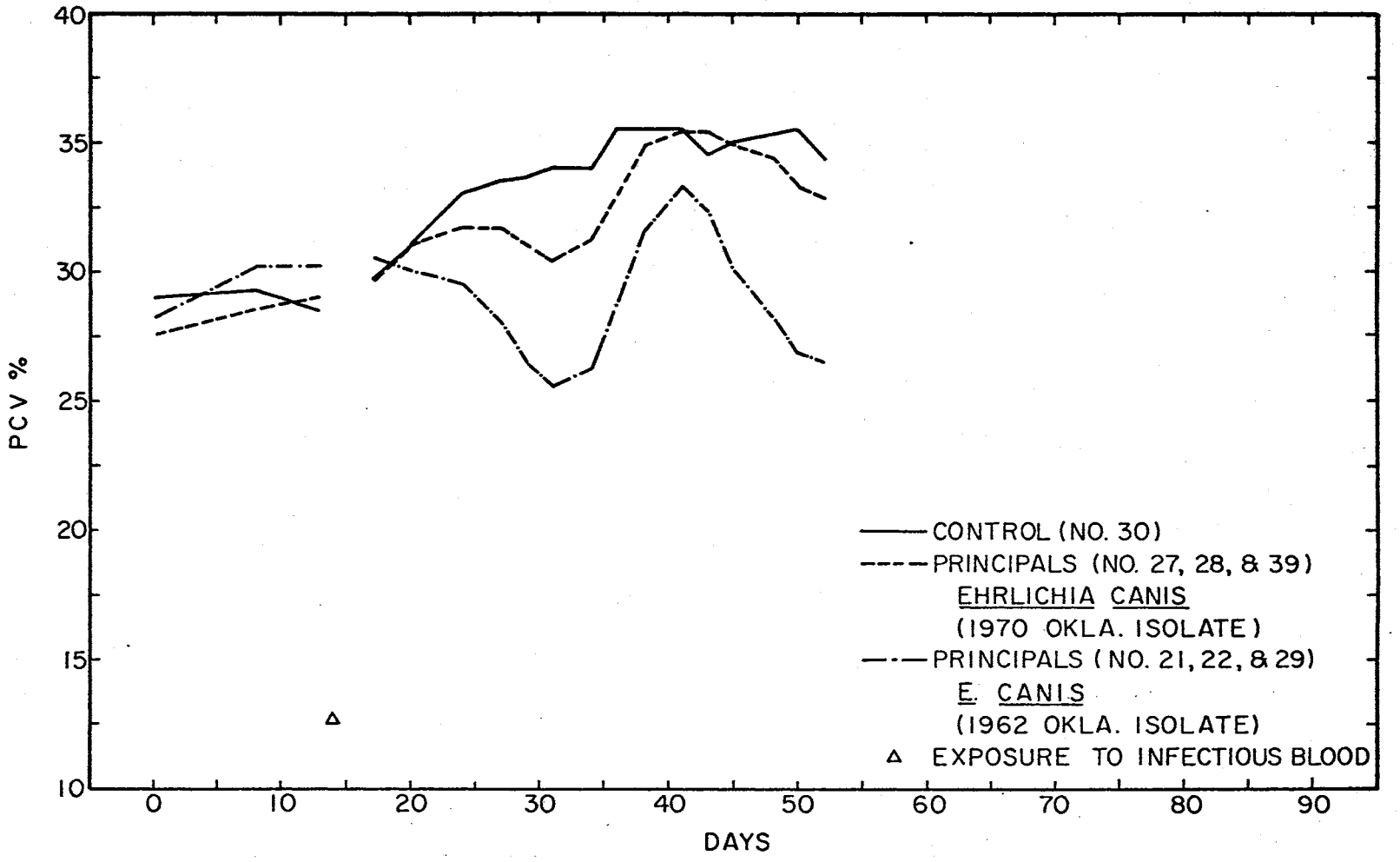

Figure 8. Moving averages of the mean packed cell volume of six principals and a control; three principals exposed to E. canis, 1962 Oklahoma isolate and three to E. canis, 1970 Oklahoma isolate

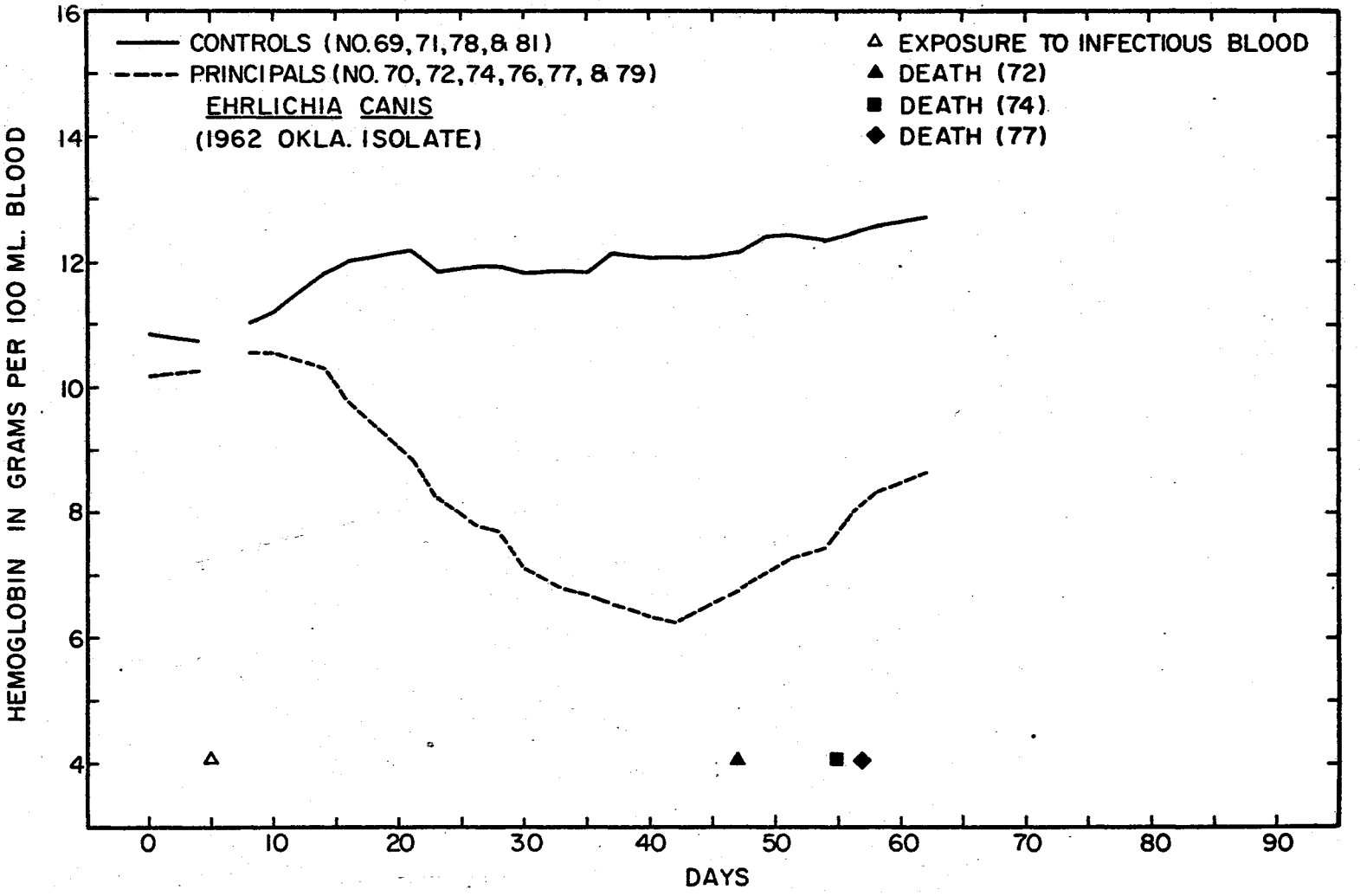

Figure 9. Moving averages of the mean hemoglobin values of six principals and four controls; principals exposed to E. canis, 1962 Oklahoma isolate

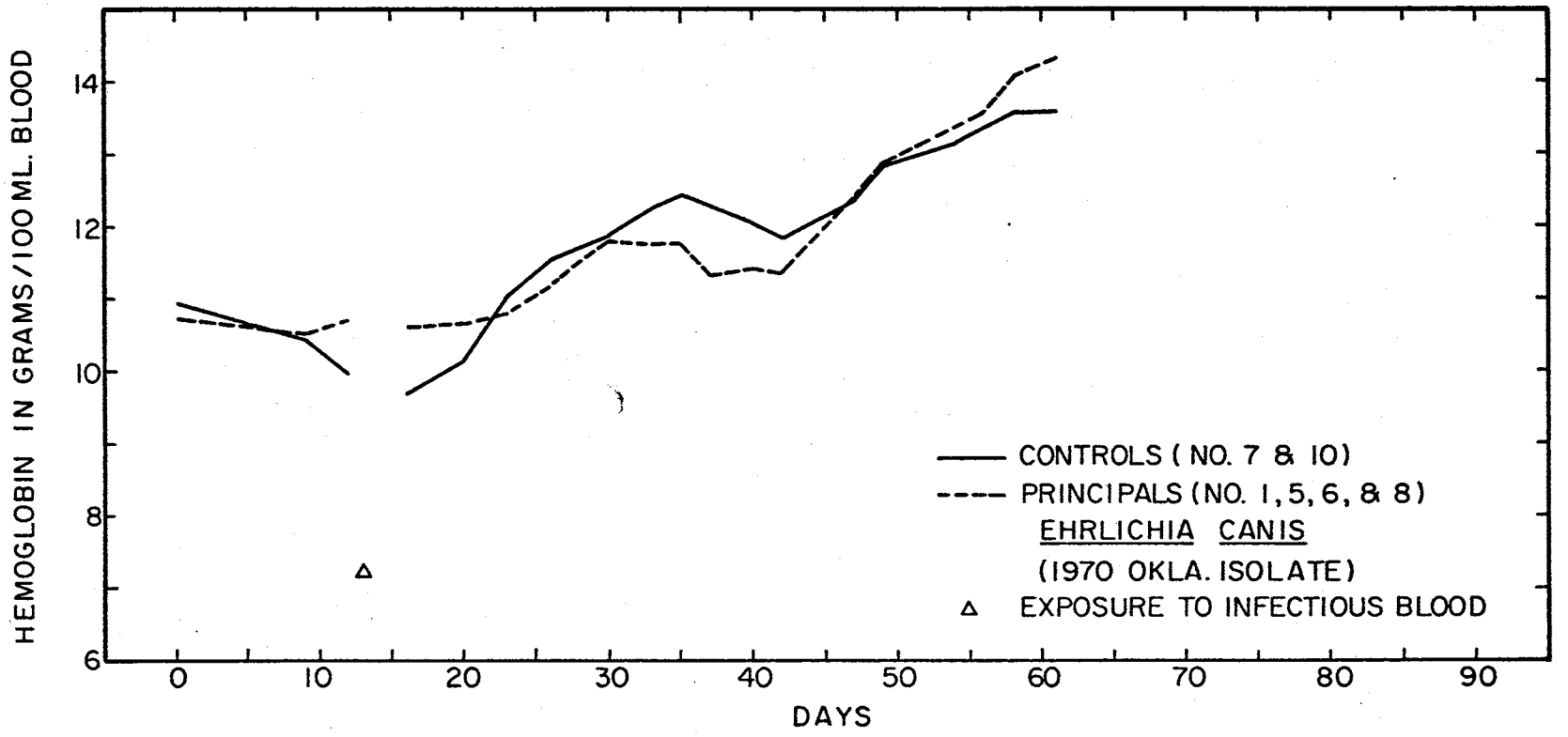

Figure 10. Moving averages of the mean hemoglobin values of four principals and two controls; principals exposed to E. canis, 1970 Oklahoma isolate

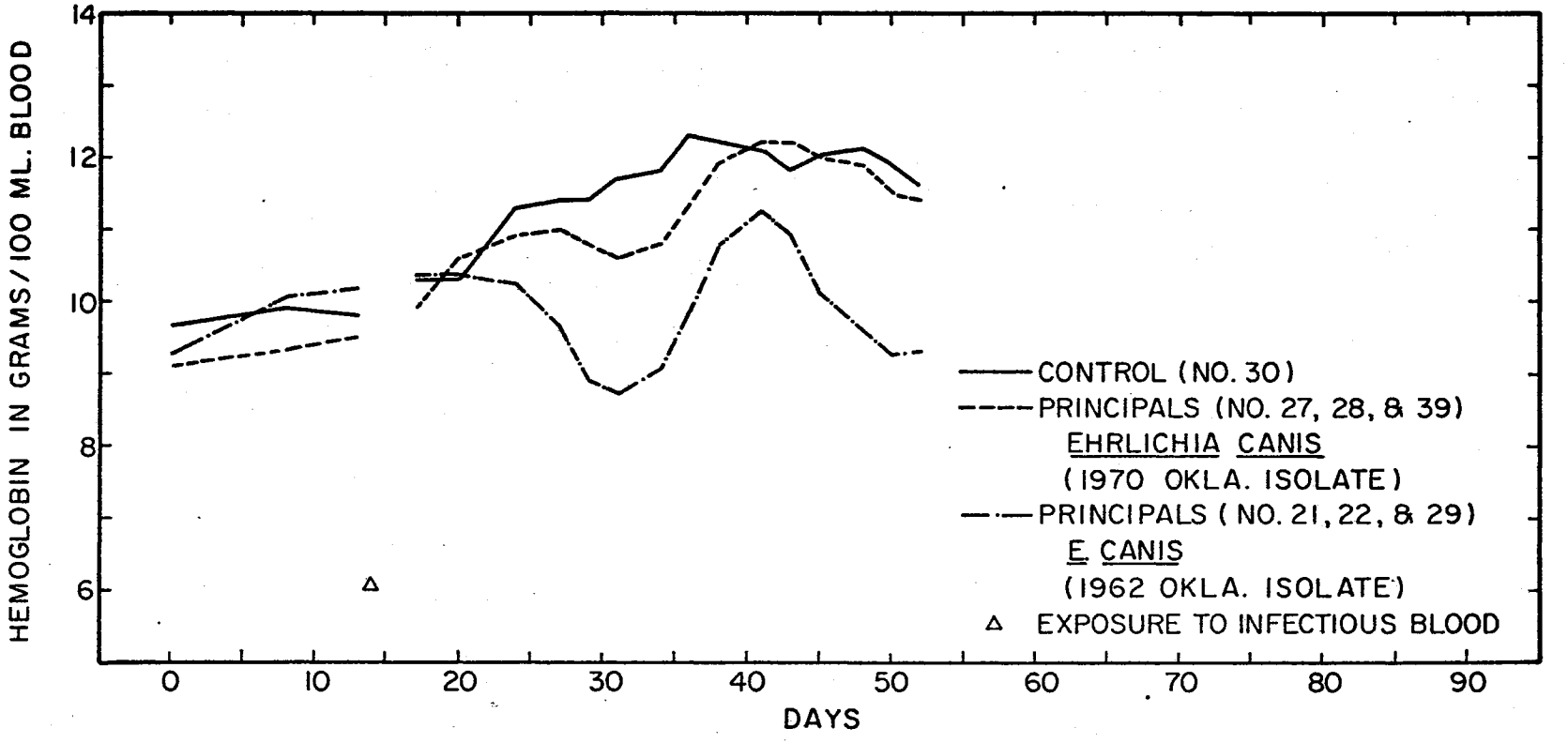

Figure 11. Moving averages of the mean hemoglobin values of six principals and a control; three principals exposed to E. canis, 1962 Oklahoma isolate and three to E. canis, 1970 Oklahoma isolate

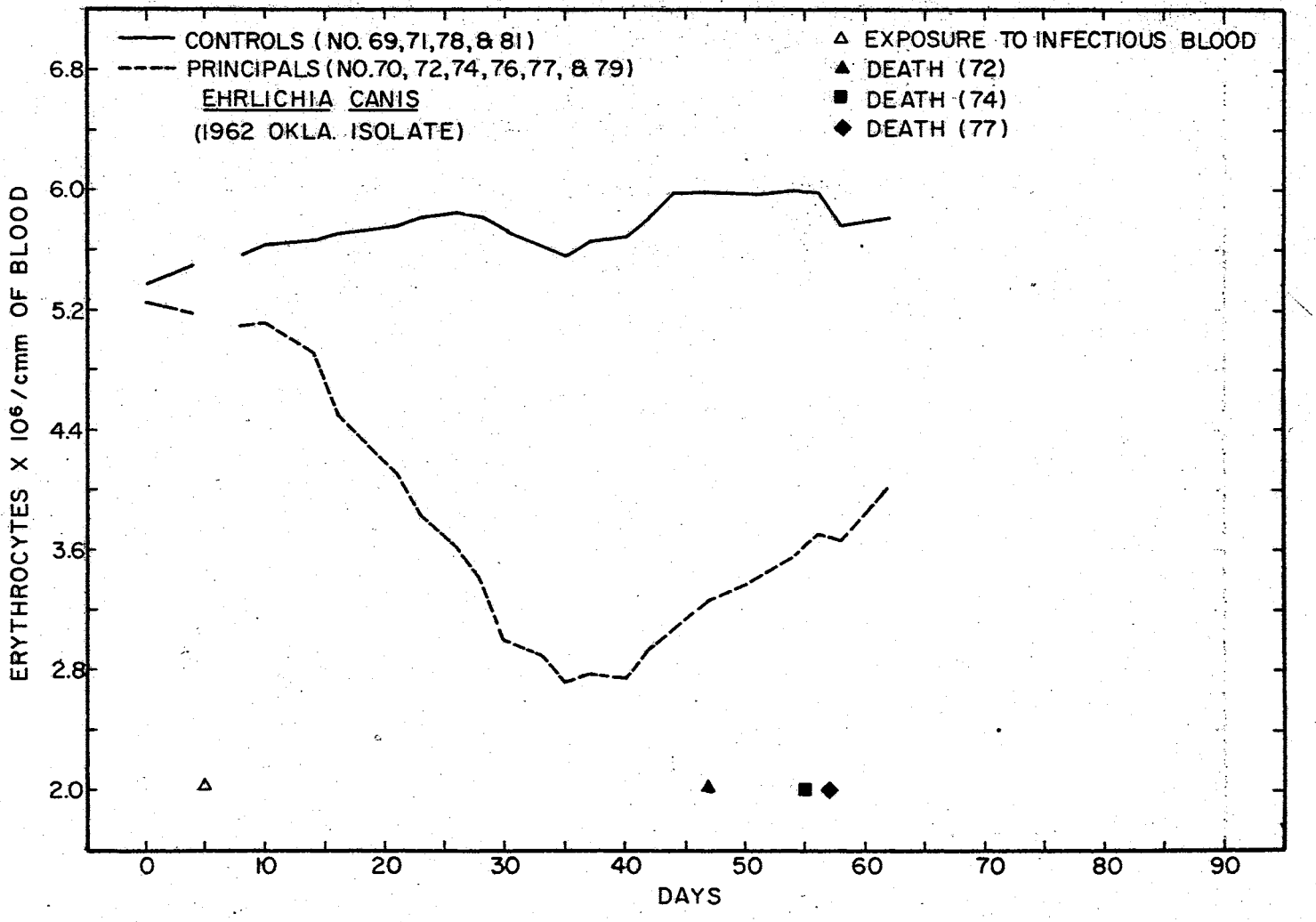

Figure 12. Moving averages of the mean total erythrocyte counts of six principals and four controls; principals exposed to E. canis, 1962 Oklahoma isolate.

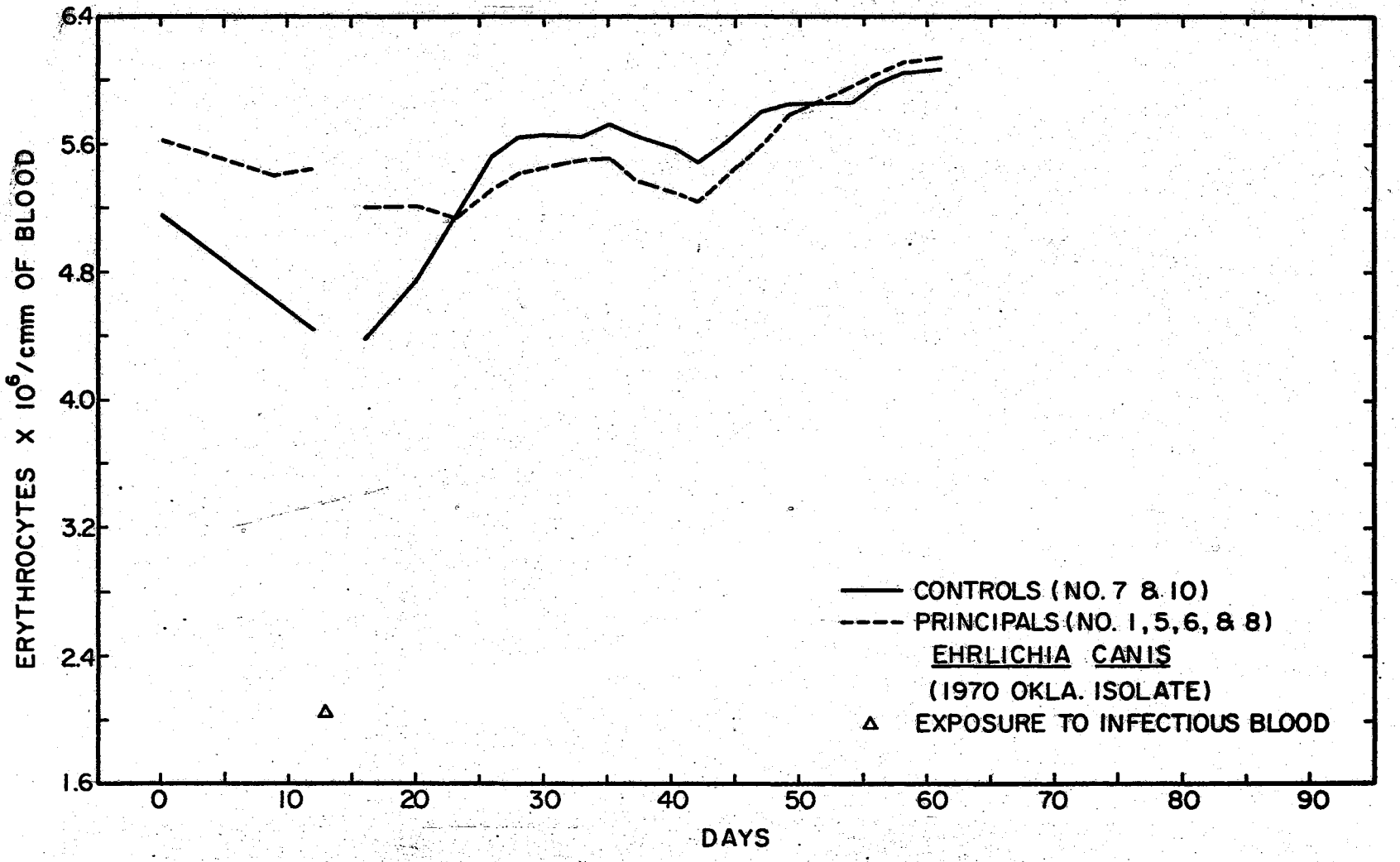

Figure 13. Moving averages of the mean total erythrocyte counts of four principals and two controls; principals exposed to E. canis, 1970 Oklahoma isolate

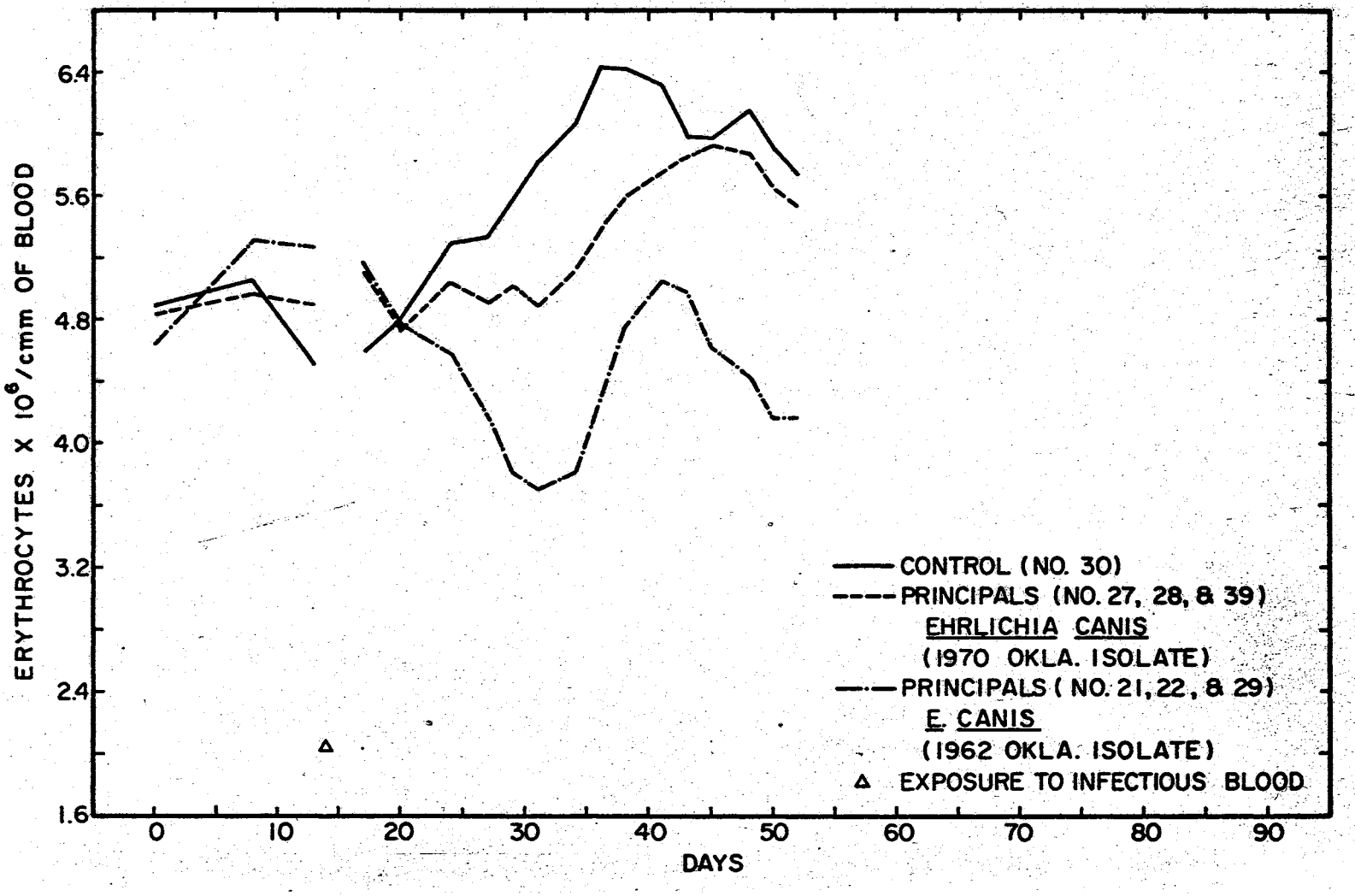

Figure 14. Moving averages of the mean total erythrocyte counts of six principals and a control; three principals exposed to E. canis, 1962 Oklahoma isolate and three to E. canis, 1970 Oklahoma isolate

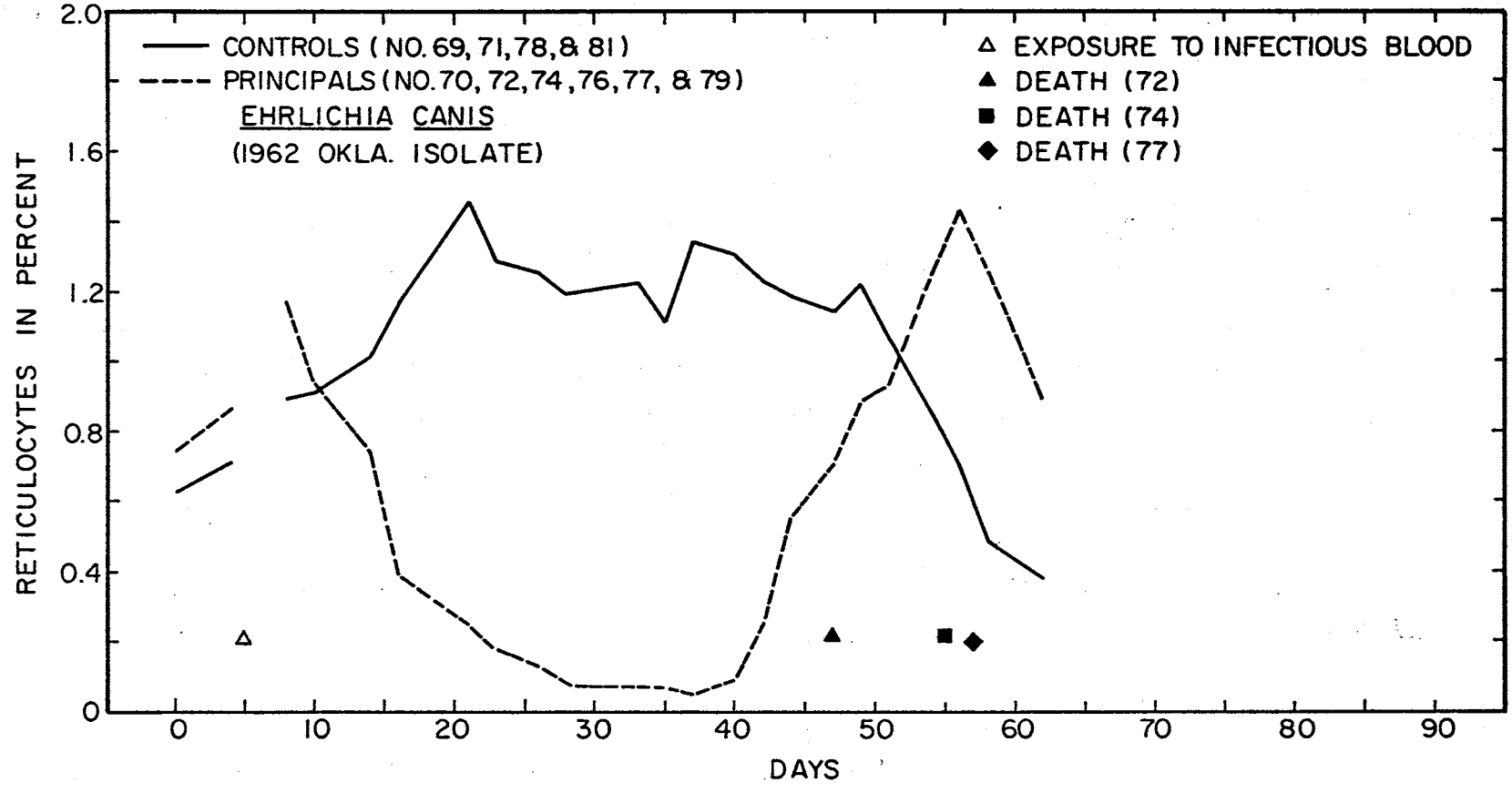

Figure 15. Moving averages of the mean reticulocyte counts of six principals and four controls; principals exposed to E. canis, 1962 Oklahoma isolate

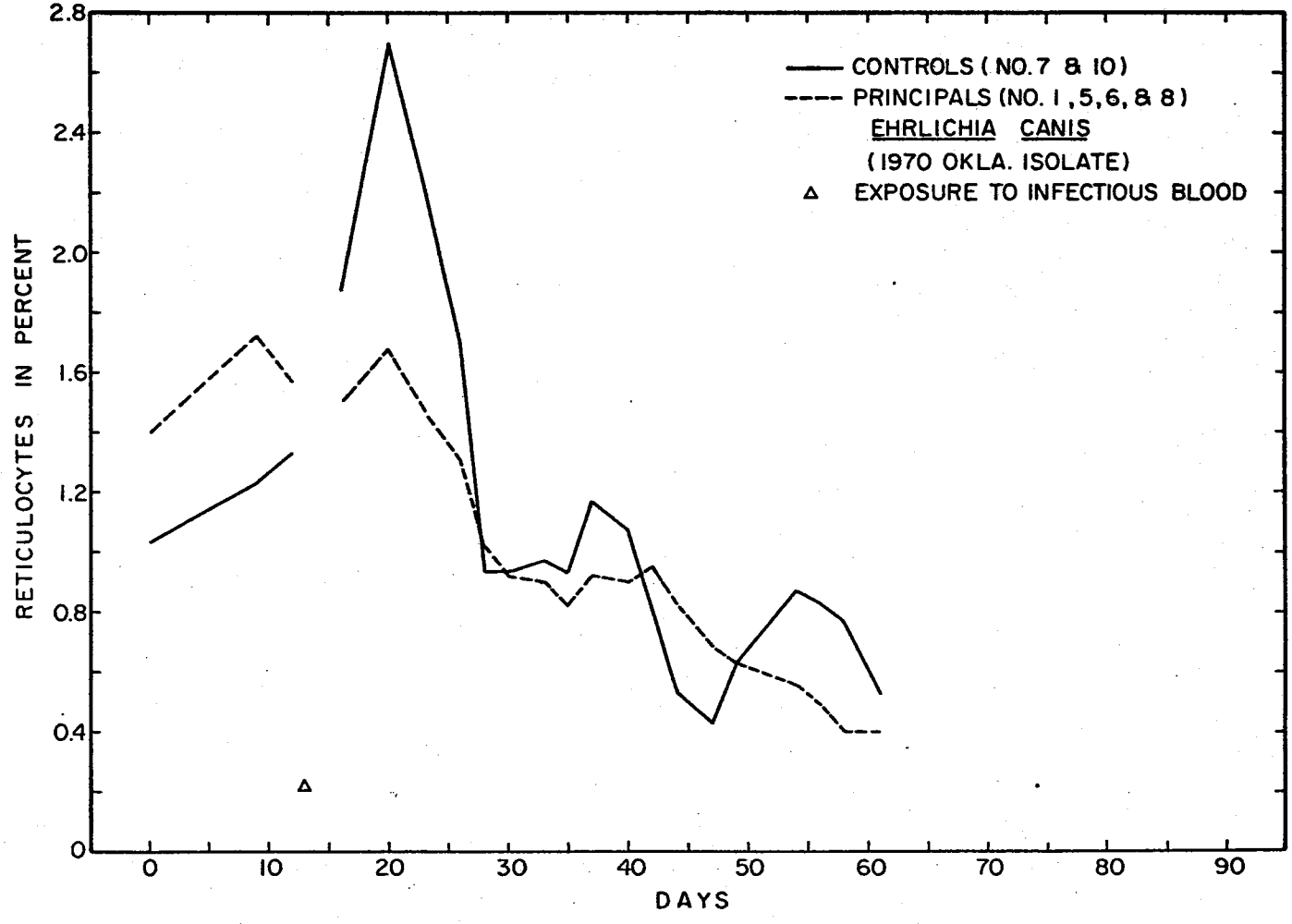

Figure 16. Moving averages of the mean reticulocyte counts of four principals and two controls; principals exposed to E. canis, 1970 Oklahoma isolate

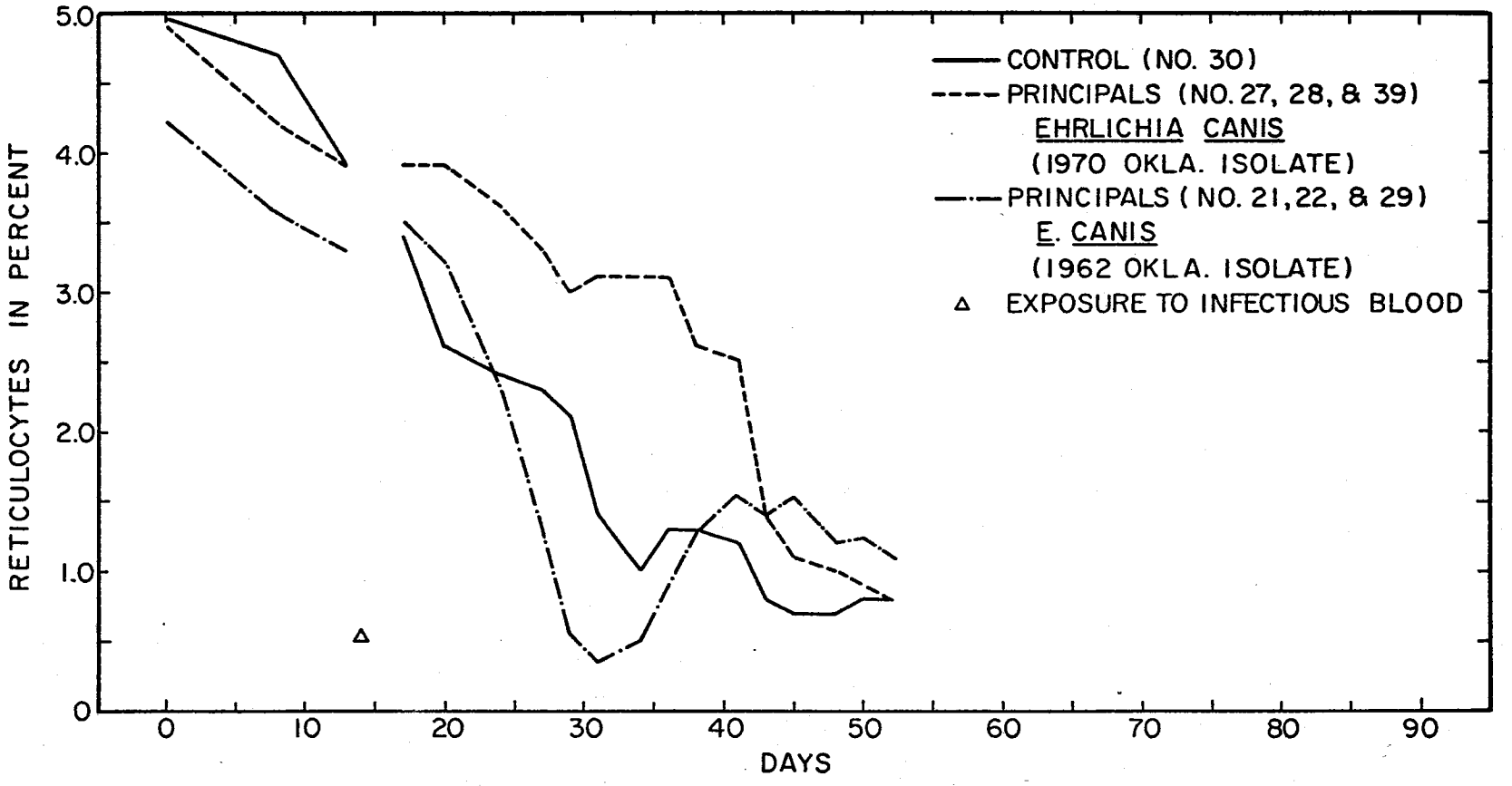

Figure 17. Moving averages of the mean reticulocyte count of six principals and a control; three principals exposed to E. canis, 1962 Oklahoma isolate and three to E. canis, 1970 Oklahoma isolate

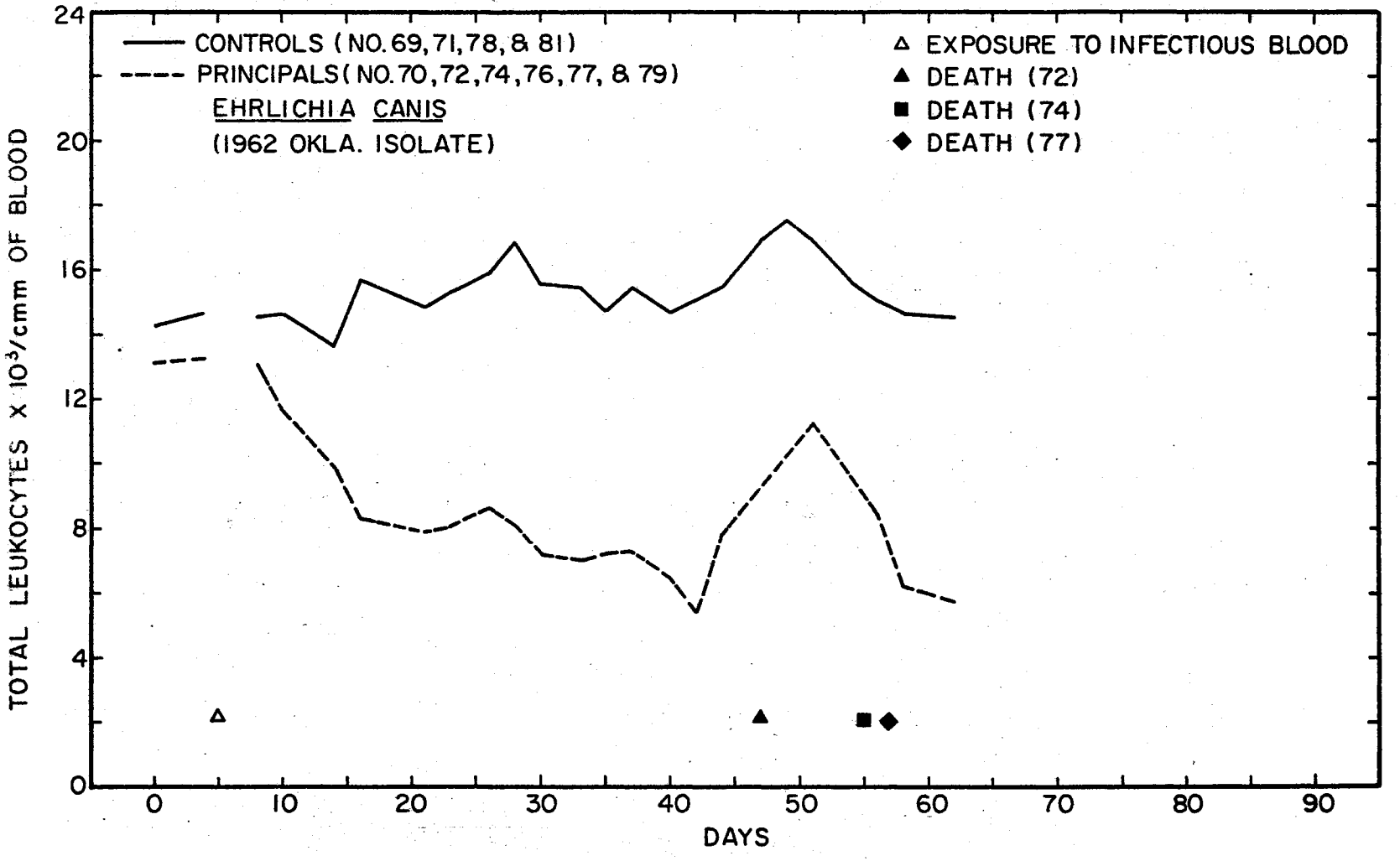

Figure 18. Moving averages of the mean total leukocyte counts of six principals and four controls; principals exposed to E. canis, 1962 Oklahoma isolate

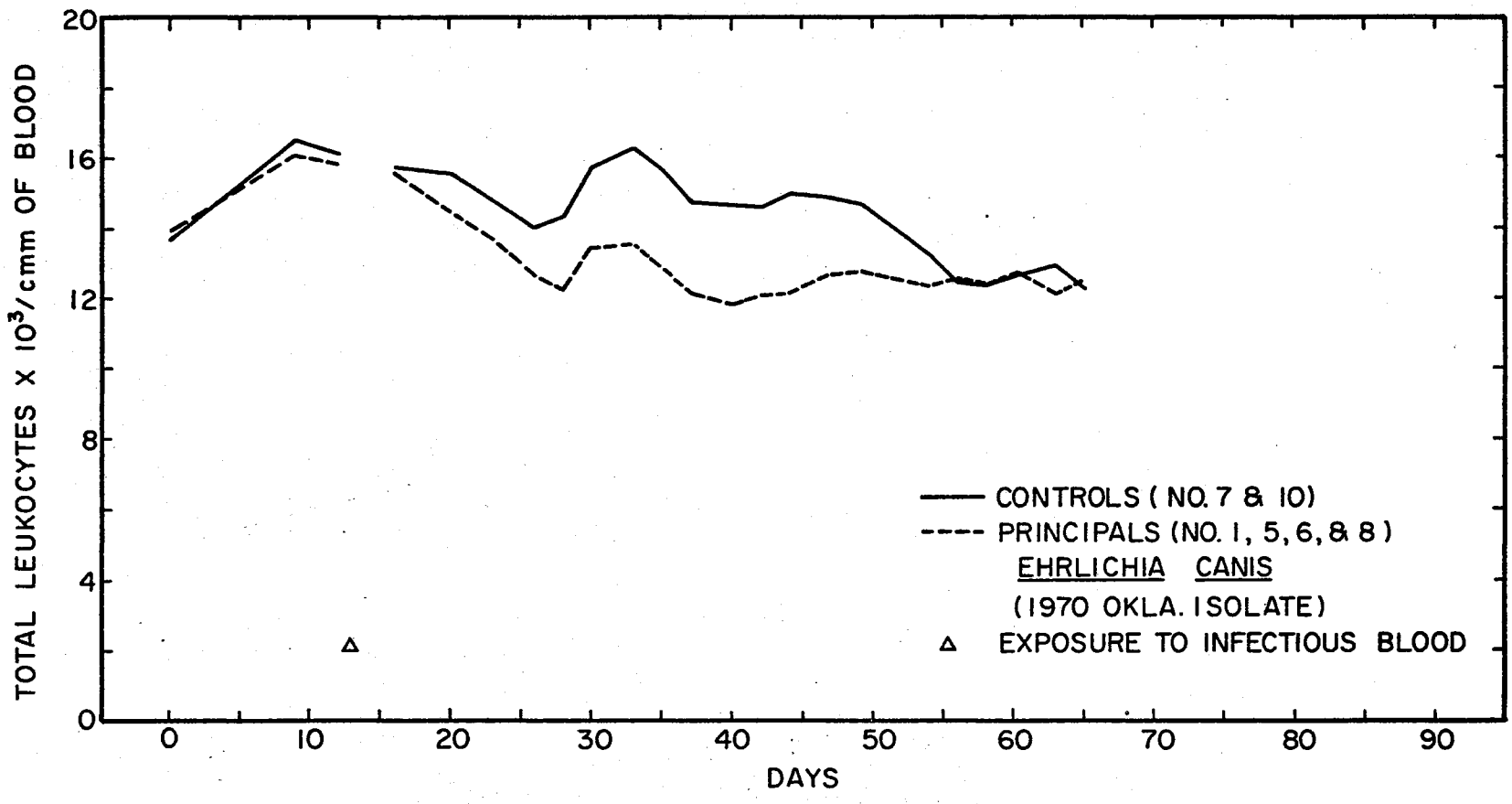

Figure 19. Moving averages of the mean total leukocyte counts of four principals and two controls; principals exposed to E. canis, 1970 Oklahoma isolate

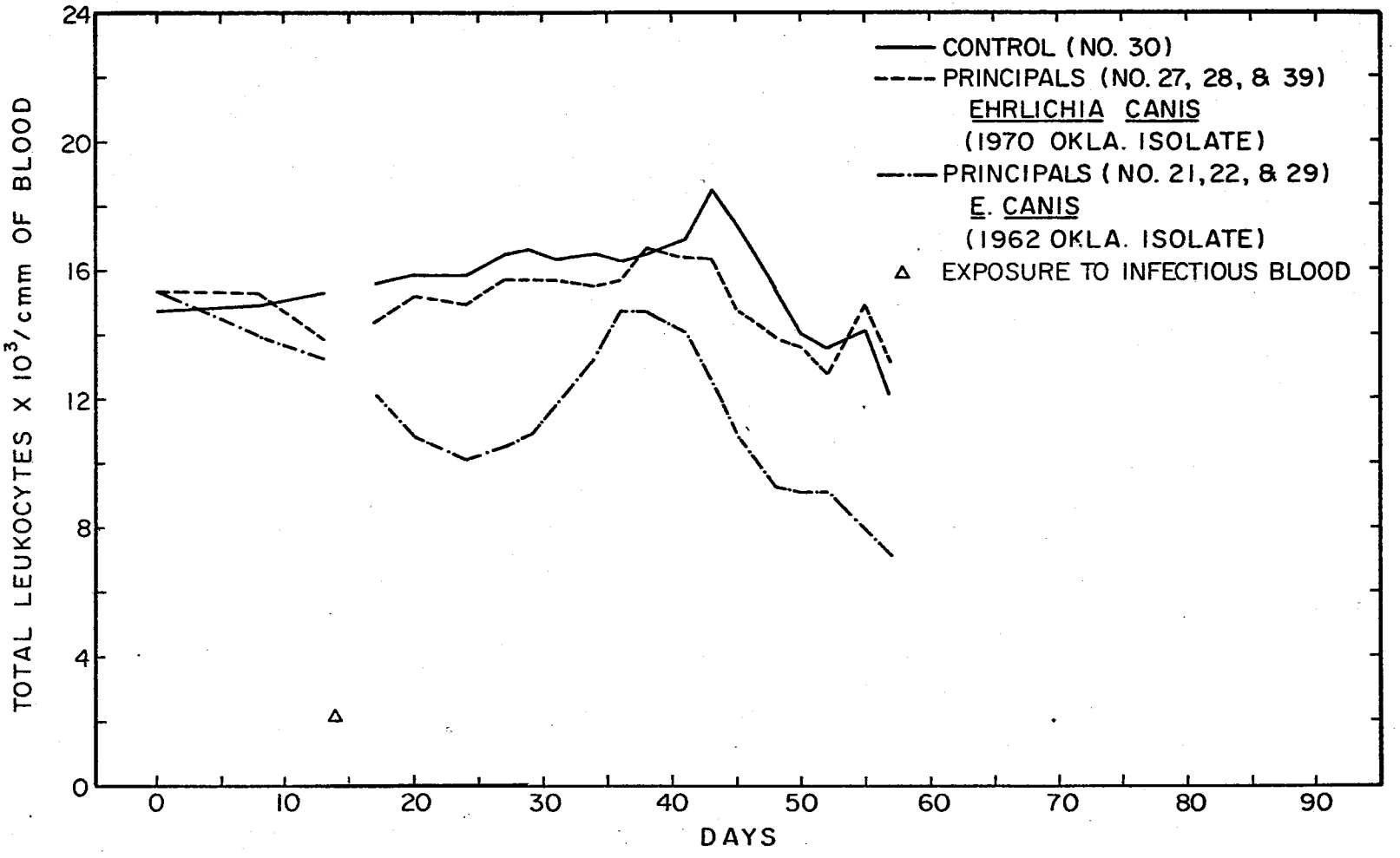

Figure 20. Moving averages of the mean total leukocyte counts of six principals and a control; three principals exposed to E. canis, 1962 Oklahoma isolate and three to E. canis, 1970 Oklahoma isolate

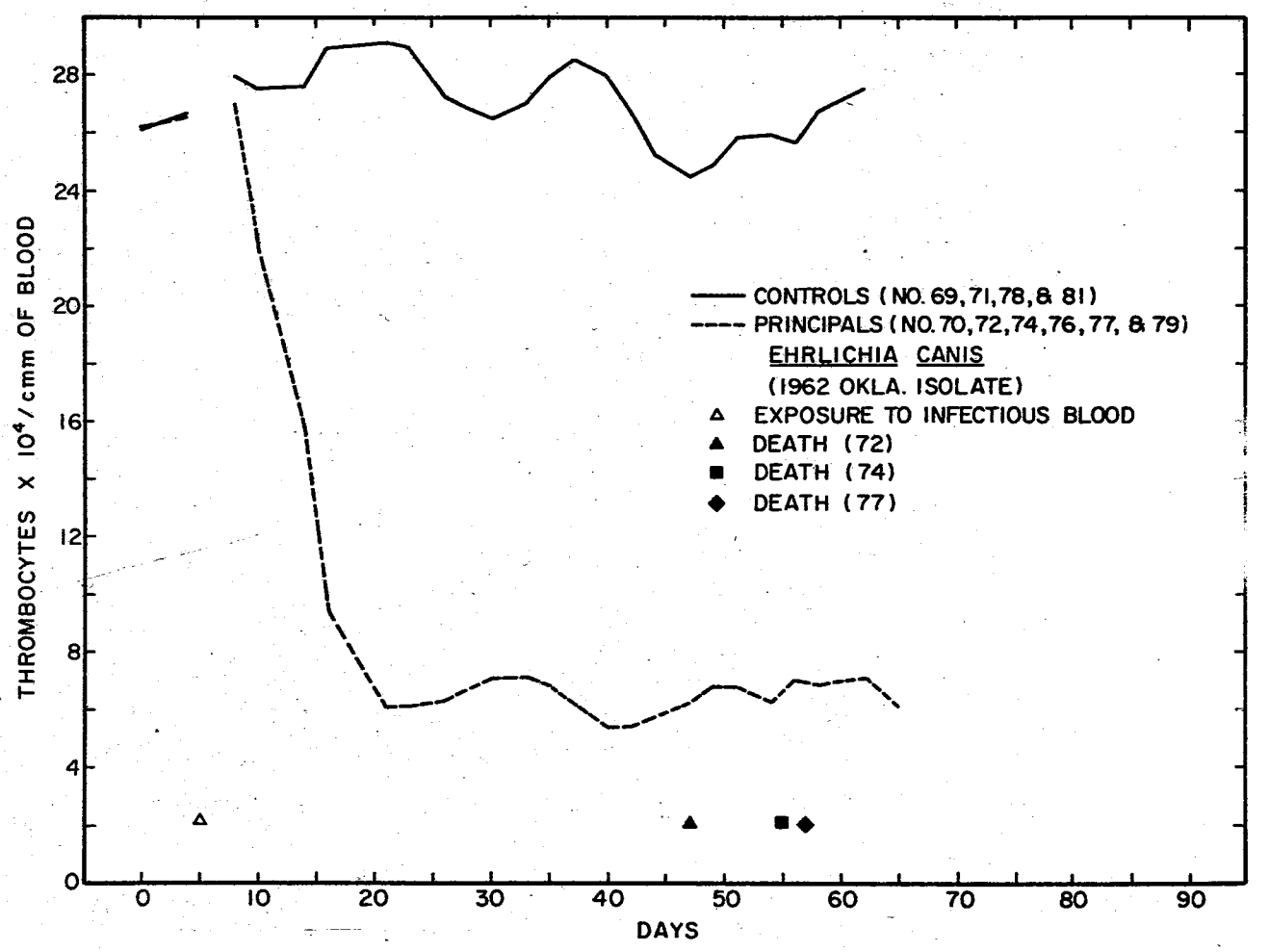

Figure 21. Moving averages of the mean thrombocyte counts of six principals and four controls; principals exposed to E. canis, 1962 Oklahoma isolate

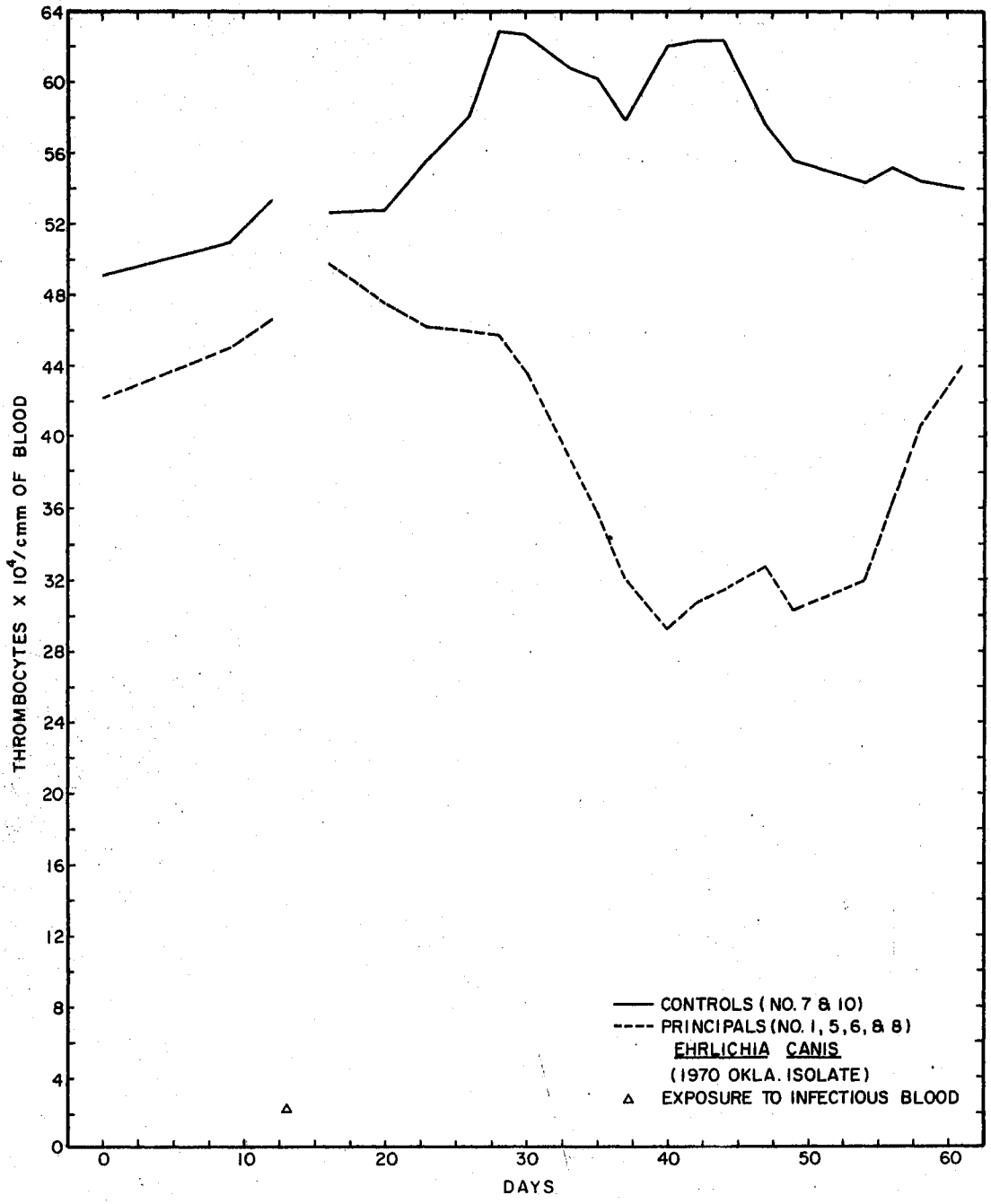

Figure 22. Moving averages of the mean thrombocyte counts of four principals and two controls; principals exposed to E. canis, 1970 Oklahoma isolate

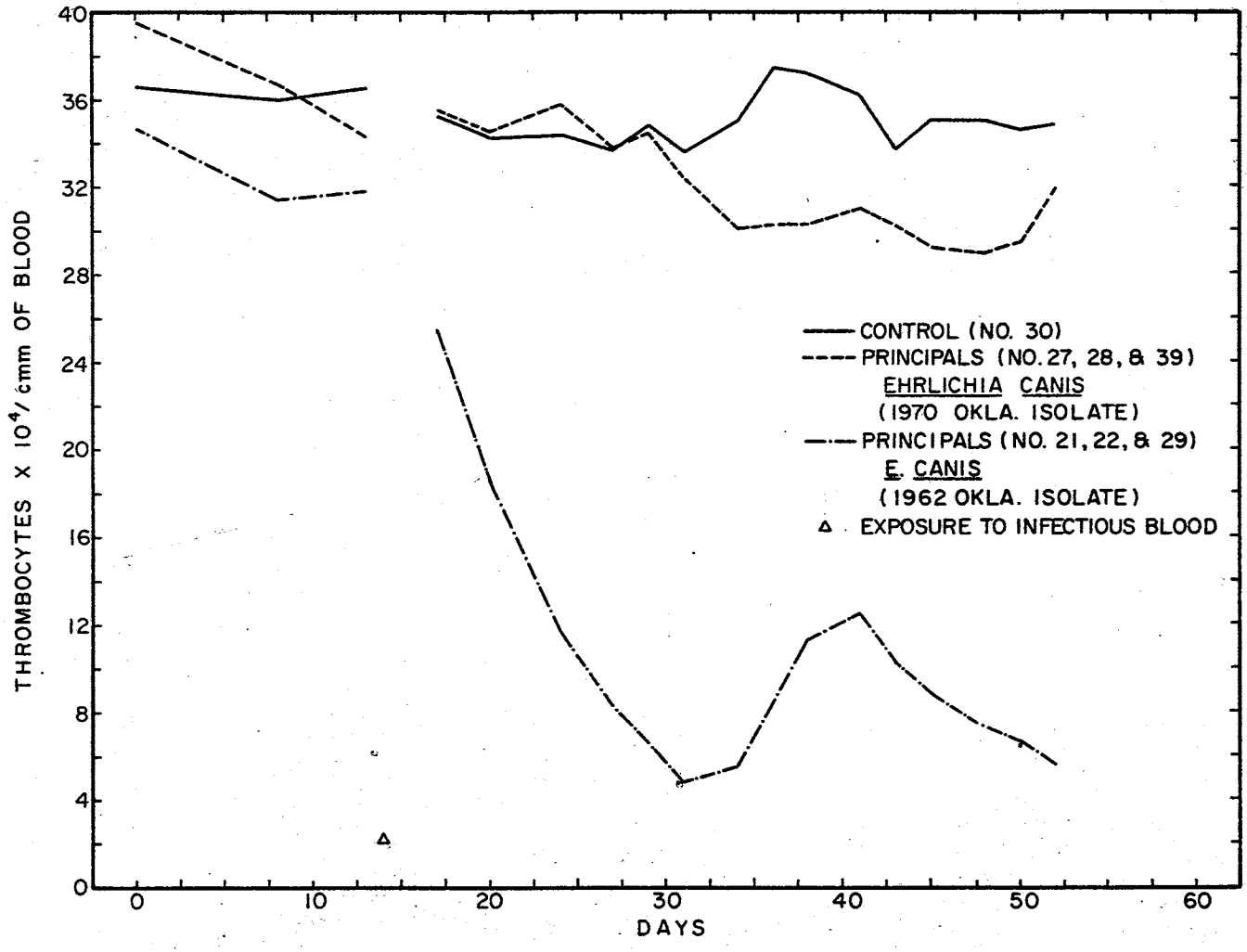

Figure 23. Moving averages of the mean thrombocyte counts of six principals and a control; three principals exposed to E. canis, 1962 Oklahoma isolate and three to E. canis, 1970 Oklahoma isolate

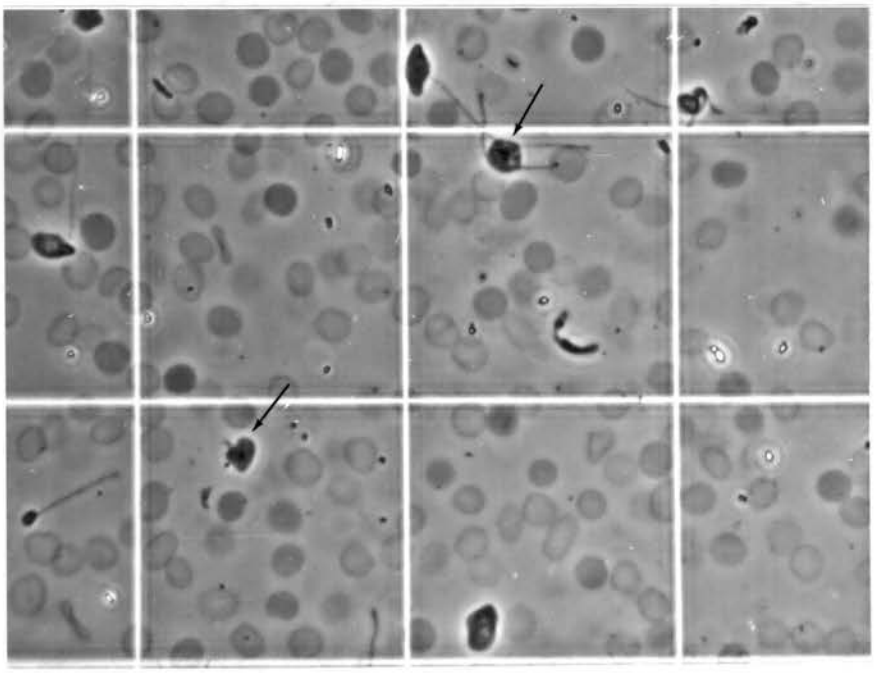

Figure 24. Thrombocytes from a pup infected with Ehrlichia canis, 1962 Oklahoma isolate. Notice decreased number and large size of thrombocytes. The photomicrograph was made thirty-six days after exposure; compare with Figure 25.

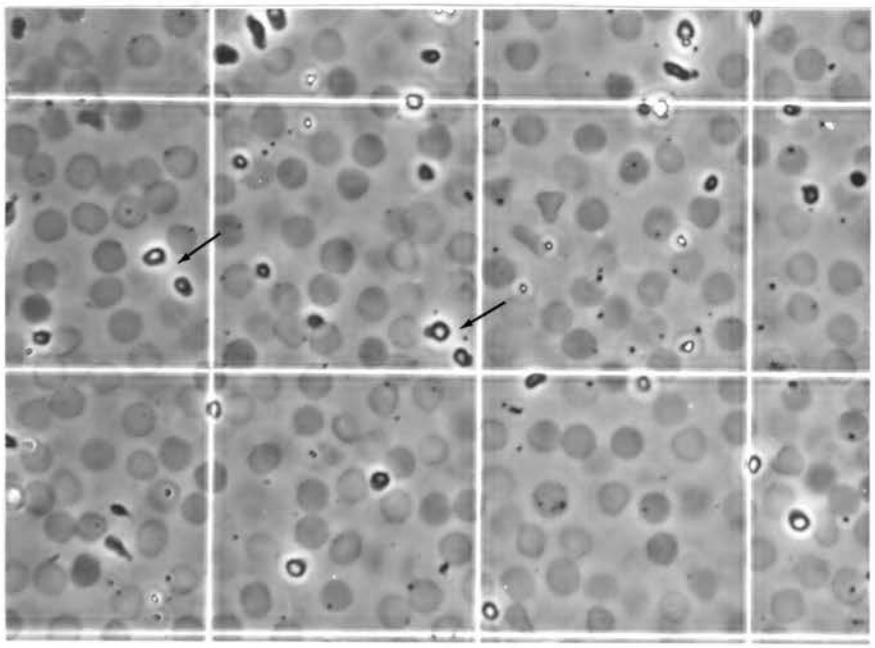

Figure 25. Thrombocytes from a littermate control thirty-six days after exposure of principals. Compare number and size of thrombocytes with those in Figure 24.

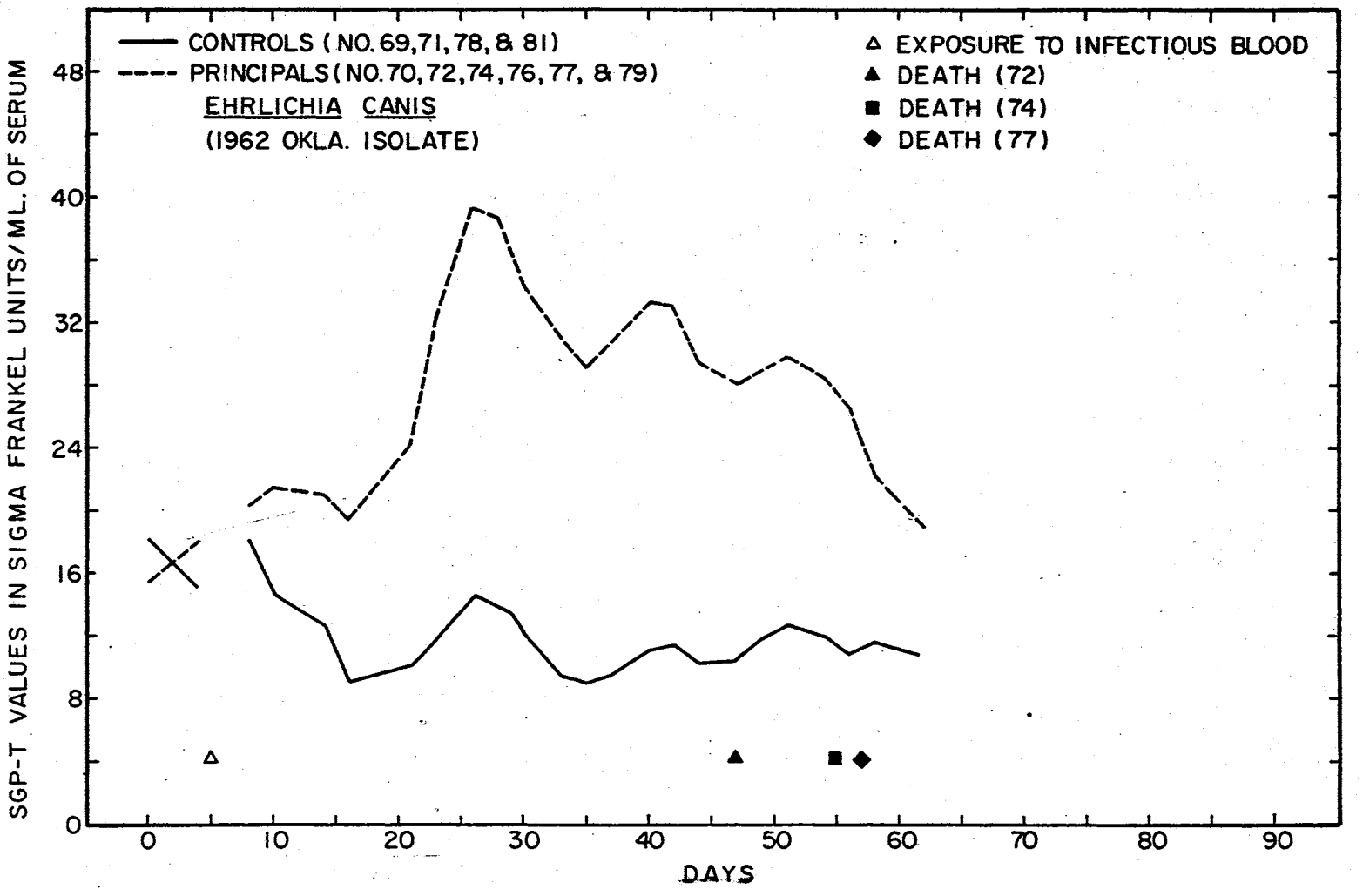

Figure 26. Moving averages of the mean SGP-T values of six principals and four controls; principals exposed to E. canis, 1962 Oklahoma isolate.

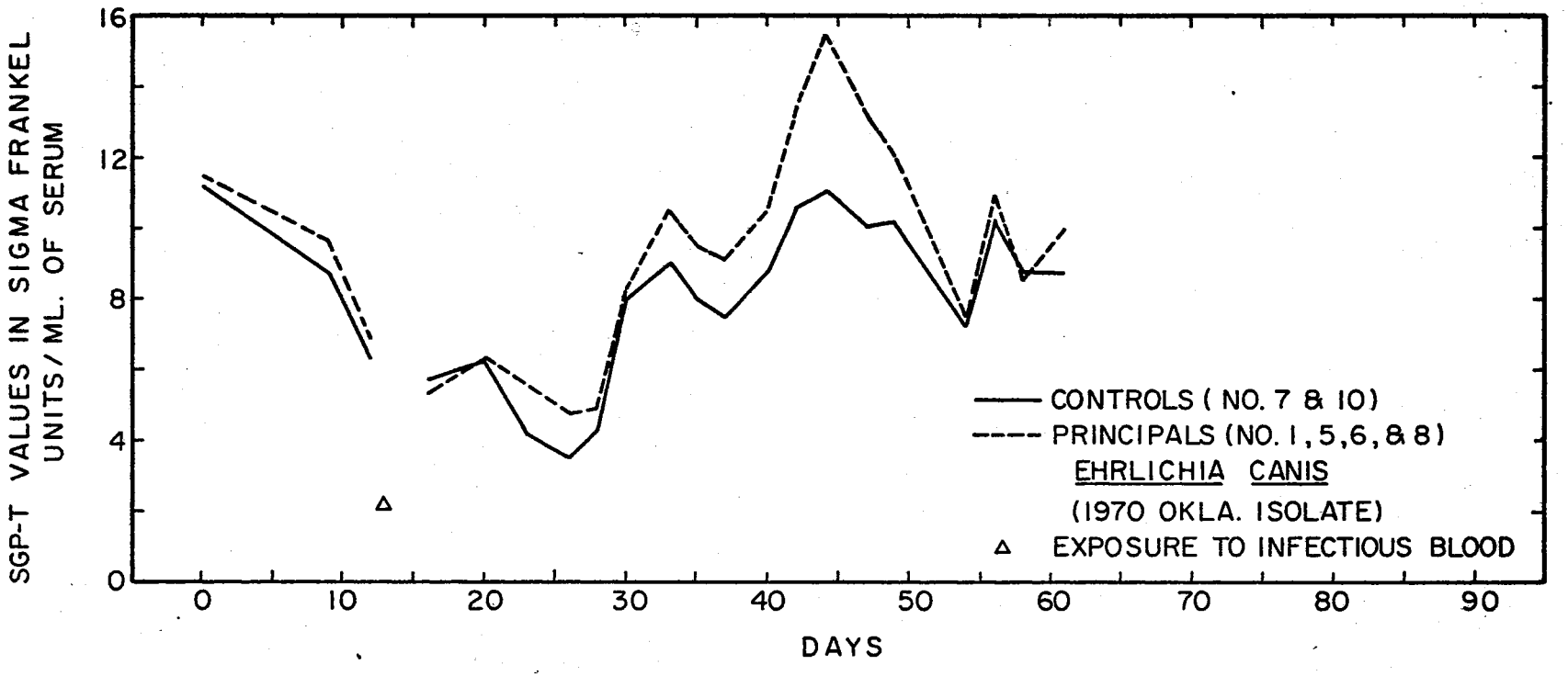

Figure 27. Moving averages of the mean SGP-T values of four principals and two controls; principals exposed to E. canis, 1970 Oklahoma isolate

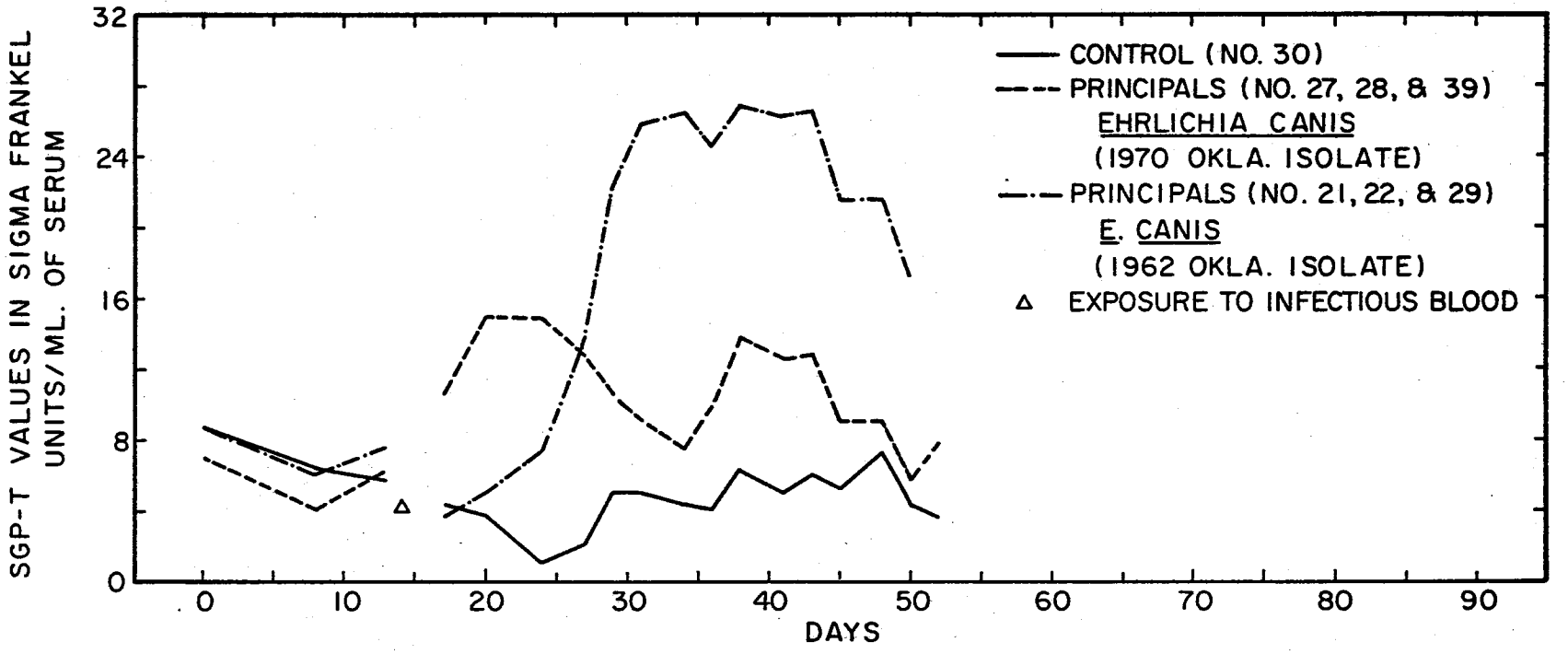

Figure 28. Moving averages of the mean SGP-T values of six principals and a control; three principals exposed to E. canis, 1962 Oklahoma isolate and three to E. canis, 1970 Oklahoma isolate

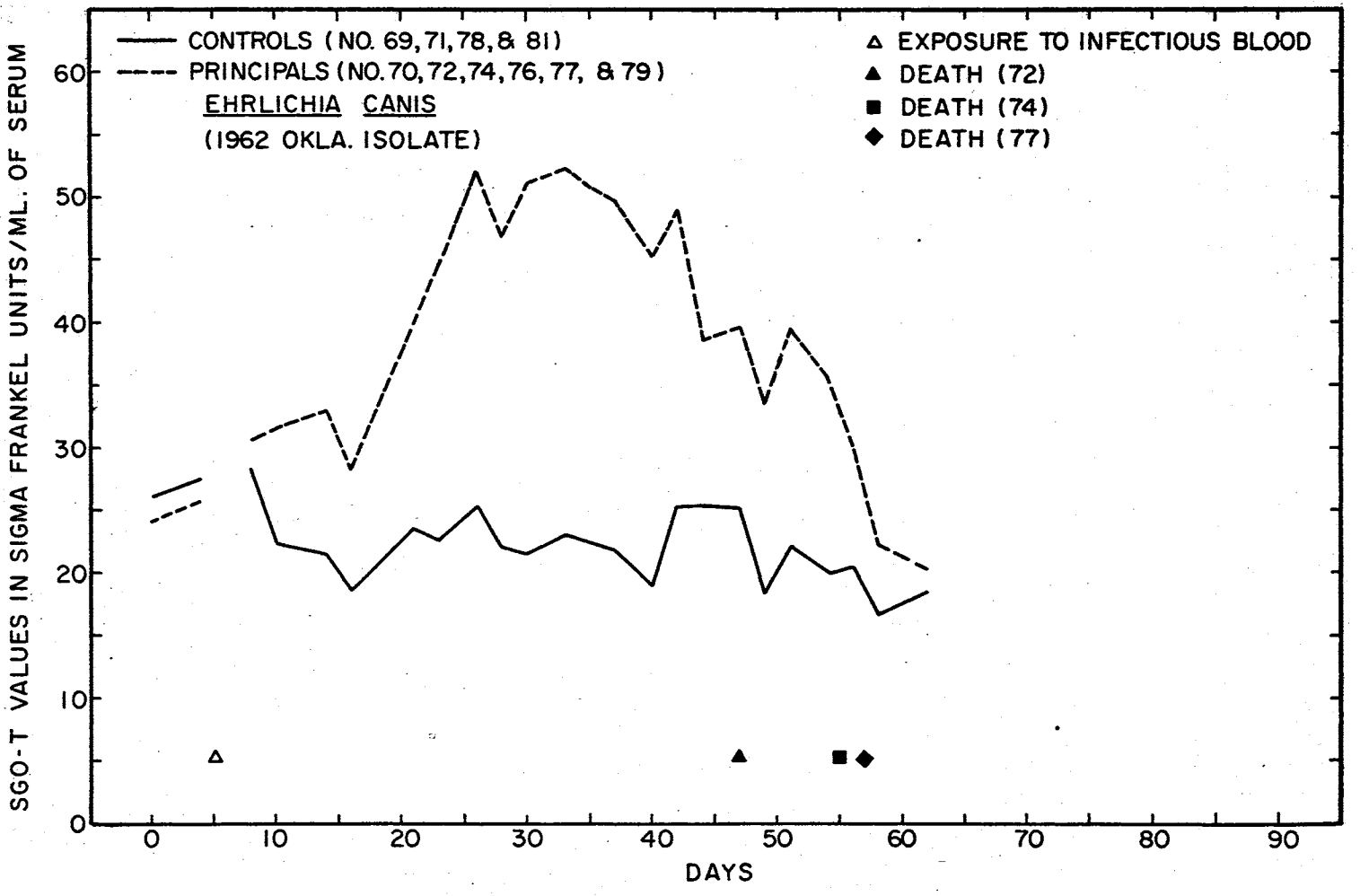

Figure 29. Moving averages of the mean SGO-T values of six principals and four controls; principals exposed to E. canis, 1962 Oklahoma isolate

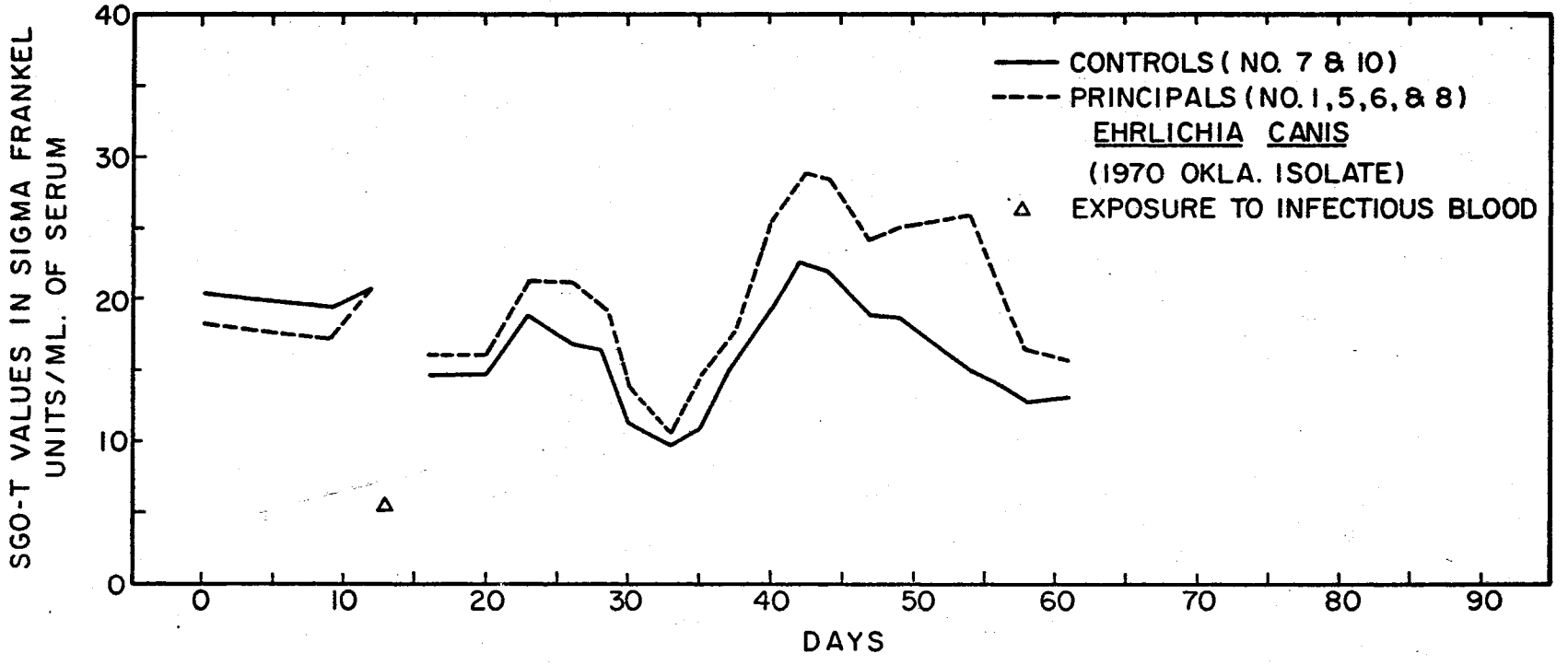

Figure 30. Moving averages of the mean SGO-T values of four principals and two controls; principals exposed to E. canis, 1970 Oklahoma isolate

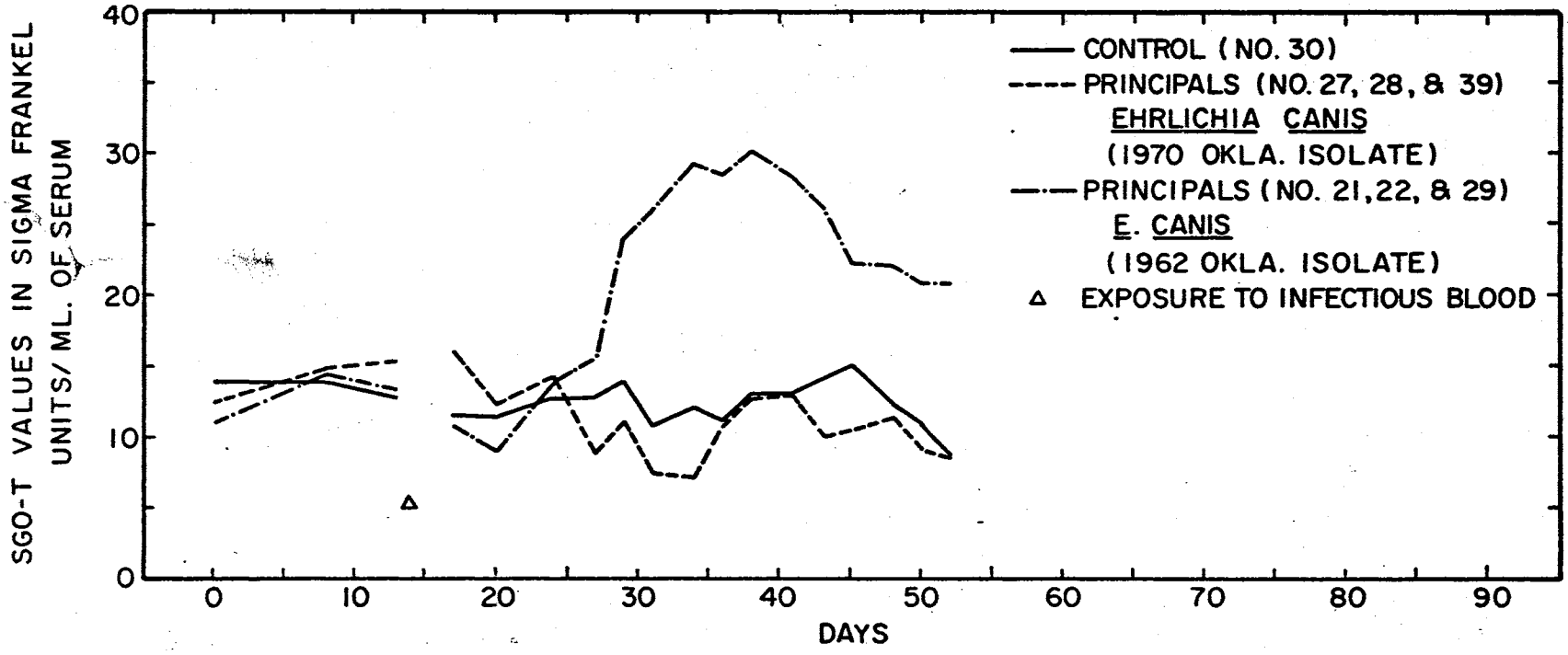

Figure 31. Moving averages of the mean SGO-T values of six principals and a control; three principals exposed to E. canis, 1962 Oklahoma isolate and three to E. canis, 1970 Oklahoma isolate

Clinical Signs

Clinical signs attendant with the infection of E. canis have been described by Ewing (1969). He stated that "...uncomplicated ehrlichiosis usually is a relatively mild disease except in young puppies in whom it may be fatal. Young animals usually have recurrent fever, often reaching 107°F. Signs of central nervous system derangement are not uncommon. Early in the course of infection, serous nasal discharge develops which later becomes mucopurulent. Photophobia usually occurs and is accompanied by bilateral ocular discharge which finally becomes purulent. Vomiting is common, and the breath usually is fetid. Splenomegaly is constant and is easily detected by palpation." Ewing (1965a) also observed papular dermatitis on the abdomen of experimentally infected dogs during the early part of the febrile period. Young pups in his study died 19 to 45 days postexposure. Huxsoll, et al., (1969), Seamer and Snape (1970), and Walker, et al., (1970) noticed epistaxis as the most common sign of an E. canis-induced syndrome which they termed tropical canine pancytopenia (TCP).

Clinical signs observed in this study were similar to those described by Ewing and Buckner (1965a) and Ewing (1969) with the exception that papular dermatitis was not observed. The temperature pattern in all the infected dogs in this study was essentially the same. For the purpose of brevity, therefore, one graph showing body temperature responses of a principal and a control animal which are representative of all experimental subjects is included. Temperature responses (Figure 3) were very characteristic showing exacerbations and remissions roughly on alternate days beginning on an average 11.83 days postexposure. Temperature reached a peak of 106 to 107°F and then gradually

dropped to lower peaks and to normal range. These findings were similar to the findings of Neitz and Thomas (1938), Lawrence (1938), Malherbe (1947 and 1948), Bool and Sutmöller (1957), and Ewing and Buckner (1965a and 1965b). In the present study a higher parasitemia was generally observed in infected dogs on the days when they had a higher temperature. Morulae were few or absent from the peripheral blood lymphocytes during the remission periods. The pattern, however, was not consistent enough throughout the study to conclude that it is a uniform feature of the disease.

Epistaxis is common in dogs suffering from TCP which presumably is caused by E. canis (Huxsoll, et al., 1969; Seamer and Snape, 1970; and Walker, et al., 1970). In this study, however, epistaxis did not occur in any dog. This difference of results probably could be explained on the basis that the two different strains of E. canis vary in pathogenicity. Since not even the taxonomic affinity between the 1962 Oklahoma isolate and the strain recovered from Southeast Asia has been resolved, any explanation would be a conjecture.

Three of the principals (#72,74 and 77) died during the experiment 42, 50 and 52 days, respectively, after exposure to infectious blood. Three other principals (#70,76 and 79) survived and apparently recovered completely.

Hematology

Bool (1959) conducted studies on the hematology of dogs infected naturally and experimentally with E. canis. In his studies the number of erythrocytes decreased to 2 million/cmm. The lowest level of hemoglobin was found to be 6 grams/100 ml. 52 days after exposure.

Erythrocyte counts and hemoglobin values then increased slowly but never reached the normal level even after 5 months, the duration of the studies. Ewing and Buckner (1965a) conducted hematologic studies on dogs with experimentally induced ehrlichiosis. They reported that packed cell volume (PCV) decreased to 9 percent 21 days after exposure in one pup and that hemoglobin values as low as 3 grams/100 ml. were observed. Erythrocyte counts decreased to as low as 1.1 million/cmm. The hematologic changes observed in their studies were quite consistent among young dogs, and striking anemia developed soon after the inoculation of the infectious blood. The reticulocyte count remained within normal range in spite of such striking anemia. Hematopoietic response was observed just before death in some animals. Their studies usually were terminated 21 days after exposure but in some cases were extended to 30 days. Anemia was also observed by Huxsoll, et al., (1970) and Walker, et al., (1970) in their study of TCP which is, as mentioned, a severe form of canine ehrlichiosis.

The hematologic changes (Figures 6,9,12,15,18 and 21) in this study were very consistent and generally were similar to the findings of Bool (1959) and Ewing and Buckner (1965a). The lowest values of hemoglobin and PCV observed were in one of the principals (#77) and were 5.5 grams/100 ml. and 15 percent, respectively. Erythrocyte counts decreased to 2.02 million in the same pup 30 days after exposure to infectious blood. When the principals are compared with the uninfected littermate controls in respect to PCV, hemoglobin values and total erythrocyte count (Figures 6,9 and 12) it is evident that severe anemia develops soon after the appearance of morulae in peripheral blood. The anemia persisted for 40 to 68 days postexposure among the

principals which survived. The dogs either died or experienced very slow recovery. The present studies were extended for a longer period than those of Ewing and Buckner (1965a) in order to observe the recovery phase of animals which survived. The anemia was not as severe as that observed by Ewing and Buckner (1965a) even though the same strain of E. canis was studied. This possibly may be explainable on the basis of reduced pathogenicity of the strain. It is appropriate to reiterate that this strain was isolated in 1962 in Oklahoma and was repeatedly passed from dog to dog without passage through Rhipicephalus sanguineus, the arthropod host. The strain may have been altered in virulence.

Reticulocyte counts (Figure 15) in all the principals remained within normal ranges in spite of severe anemia, indicating that the hematopoietic capabilities of the hosts were impaired. Three pups (#72,74 and 77) did not show hematopoietic response before death, findings contrary to those of Ewing and Buckner (1965a). In their study a slight hematopoietic response occurred just before death. Reticulocyte counts in the three pups which survived, and finally recovered, increased slowly beginning from 36 to 38 days after the exposure. This finding indicates that the hematopoietic tissue is no longer suppressed and is, in fact, stimulated at this phase. The erythrocytic needs of the animal are slowly restored.

Bool (1959) observed leukopenia soon after exposure but then leukocytosis developed during the first phase of fever. McGaughey, et al., (1962) observed monocytosis in their studies. Ewing (1969) stated that "...leukopenia occurs early in the course of canine ehrlichiosis but usually is followed by monocytosis." Walker, et al.,

(1970) observed very marked leukopenia among dogs infected with TCP. Many dogs with epistaxis at the time of death had total leukocyte counts below 1,000. Furthermore, the leukopenia observed by Walker, et al., (1970) was balanced, an unusual finding in canine ehrlichiosis, which is not substantiated by others. In the present study leukopenia was observed consistently in all the principals (Figure 18) while leukocyte counts in control pups remained within normal ranges. This observation is similar to the findings of Bool (1959), Ewing (1969), and Walker, et al., (1970). However, leukopenia was not as marked as described in some cases by Walker, et al., (1970). The decrease in the leukocytic count started soon after exposure to infectious blood and severe leukopenia developed, on the average, 36 days after exposure. Leukopenia persisted throughout the experiment in surviving principals, a fact contrary to the findings of Bool (1959) who stated that the number of leukocytes decreased slightly in the beginning but rose during the first phase of fever to 15-20,000. No explanation is obvious for these contrasting results; however, difference in breeds of dogs and in strains of E. canis may be important factors.

Of the three pups (#72,74 and 77) which succumbed, high leukocytic counts were observed before death. This was thought to be due to a bacteremia because of neutrophilia which occurred 39 to 44 days after exposure, 2 to 5 days before death. Lymphopenia and slight monocytosis were seen in all the principals beginning, on the average, 28.5 days postexposure and were seen throughout the remainder of the study; the counts in littermate controls remained within normal ranges. This observation agrees with McGaughey, et al., (1962) and Ewing (1969 and 1970) but contrasts with Walker, et al., (1970). In

the latter studies balanced leukopenia occurred in all the infected dogs.

The mechanism governing the development of lymphopenia and monocytosis is not known. One possible explanation is that the life span of lymphocytes is reduced and the pace of production is lower than the pace of destruction. Although definite evidence supporting this hypothesis was not found, many lymphocytes were observed to be of irregular shape.

Carmichael and Fiennes (1942) and McGaughey, et al., (1962) observed an increase in the number of thrombocytes in early stages of rickettsiosis (ehrlichiosis) of dogs. Foster and Cameron (1968) reported severe thrombocytopenia among sheep experimentally infected with tick-borne fever. They stated that "...the ensuing thrombocytopenia was however of relatively short duration, the platelet counts returning to normal within 7 to 10 days." Severe thrombocytopenia was observed in dogs in Tunisia by Bobin, et al., (1962). Although the dogs were suffering from a disease of unknown etiology, Huxsoll, et al., (1970a and 1970b) later suggested that it was caused by E. canis. Huxsoll, et al., (1970a and 1970b) also observed severe thrombocytopenia which was usually associated with epistaxis and other forms of hemorrhage in experimentally infected German Shepherds as early as 10 days and up to 120 days after exposure to blood containing E. canis.

In the present study a severe thrombocytopenia (Figure 21) developed 9 to 14 days after exposure and was observed consistently in all the principals. In one pup (#79) the thrombocyte count decreased to 21,000/cmm. Thrombocytopenia persisted throughout the study, a period of 4 months in 2 pups (#70 and 79). These findings are quite in agreement with Bobin, et al., (1962), Foster and Cameron

(1968), and Huxsoll, et al., (1970a and 1970b) but are completely at variance with Carmichael and Fiennes (1942) and McGaughey, et al., (1962). In light of severe bone marrow depression usually noticed in canine ehrlichiosis, increase in the number of thrombocytes cannot be expected. By carefully reviewing the papers of Carmichael and Fiennes (1942) and McGaughey, et al., (1962), it seems probable that these two groups of workers were dealing with some organism other than E. canis. This seems evident both from their description and from the photomicrographs they published. Carmichael and Fiennes (1942) described the organism and stated that "...Rickettsia appear in the cytoplasm as coccoid or bacilliform bodies, stained a characteristic crimson color; the color is quite different from that of eosinophilic granules. The bacilliform bodies are small and usually arranged in clumps, sometimes in projections of the cytoplasm having the appearance of pseudopodia; they may be very minute or about the size of a coliform bacillus." McGaughey, et al., (1962) observed the organism in the form of a minute dot, dash or ellipsoidal configuration. In both instances, perhaps it is not unjustified to conclude that these workers were dealing with some organism other than E. canis and that it caused an increase in the number of thrombocytes.

Epistaxis and other hemorrhages were not associated with thrombocytopenia in this study. Severe epistaxis, accompanying thrombocytopenia, was observed commonly in Southeast Asia by Huxsoll, et al., (1970a and 1970b) and Walker, et al., (1970). No definite statement can be made concerning the difference in effect of the 1962 Oklahoma isolate and those strains of E. canis studied in military dogs in Asia. Severe thrombocytopenia is produced in both

instances, but hemorrhages have never been seen in pups infected with the 1962 Oklahoma isolate. Military dogs are working dogs, and the nature of work could play an important role, e.g., by making them more vulnerable to traumatic injury. Durand (1961, cited by Walker, et al., 1970) has suggested that work habits may have an important role in determining the development of epistaxis. Durand (1961) also stated that civilian hunting or racing dogs whose work habits nearly parallel those of military dogs have been seen to develop severe epistaxis in Tunisia. Another possible explanation for the severe nature of ehrlichiosis in Southeast Asia is that it may be complicated by other unrecognized factors such as inapparent infectious agents.

Most of the thrombocytes in the principals in this study were almost double the size of those in their littermate controls (Figures 24 and 25). The increase in size was first observed in the principals 10 to 15 days postexposure and persisted throughout the remainder of the experiment. No other workers have reported such a finding, and the significance of this increase in platelet size is not known.

Prothrombin time was found to be within normal ranges in all the principals and there was no difference between them and their littermate controls. This finding is in agreement with Huxsoll, et al., (1970a and 1970b) and Walker, et al., (1970) who studied prothrombin times in an effort to determine why epistaxis and other hemorrhaging occurred in dogs with TCP.

Chemical Studies

Detailed studies on liver and kidney functions

of dogs with ehrlichiosis have not been reported by earlier workers. Walker, et al., (1970) conducted investigations on tropical canine pancytopenia and found that bilirubin values were within normal ranges; alkaline phosphatase and SGO-T values were above normal limits on a few occasions, but results were not statistically significant.

Liver Functions. Bilirubin values in this study were always within the normal range, indicating that the anemia observed was not of the hemolytic type.

Serum glutamic pyruvic transaminase and serum glutamic oxalacetic transaminase values were determined and are recorded in Figures 26 and 29. The principals experienced an increase in the SGP-T and SGO-T values, but the controls remained normal. Values then decreased to the normal levels in all principals 40 to 45 days after exposure. In some of these principals (#70,72 and 77) SGP-T values occasionally ranged from 60 to 75 Sigma Frankel units 3 to 5 weeks after exposure. These values, according to Cornelius and Kaneko (1963), lie within the lower margin of slight liver necrosis. Hildebrandt, et al., (1970) observed focal necrosis of the liver in histopathologic studies of TCP. In the light of our observation of slightly elevated SGP-T and SGO-T values and the statement of Hildebrandt, et al., (1970) it can be concluded that slight liver necrosis is produced in canine ehrlichiosis. The mechanism of liver damage by E. canis is not known and was not determined in this study. Although E. canis has been constantly seen in mononuclear cells in impression smears prepared from the liver, by Huxsoll, et al., (1970b), their direct effect on liver parenchymal cells is not known.

Kidney Fucntions. Blood urea nitrogen (BUN) and creatinine values in the principals were not found to be different from their littermate controls except in one pup (#79) which had 21 mg./100 ml. and 30 mg./100 ml. of serum, 29 and 33 days after exposure, respectively. Glenn (1968) stated that common BUN values in young dogs are between 15 and 25 mg./100 ml. of plasma but, at times, go as high as 30 mg. In our studies BUN values of 21 and 30 mg. do fall within this range and could, therefore, be classified as normal. The BUN values among principals in our study tend not to agree with those of Walker, et al., (1970) who found significantly elevated values during the terminal phases of TCP. They stated that "...it was evident that after several months of illness, the BUN content was the best prognostic index." Walker, et al., (1970) reported that BUN values generally increased after constant work. Panciera (1968, cited by Ewing 1969) observed petechiae in the renal cortices of dogs experimentally and naturally infected with the 1962 Oklahoma isolate of E. canis. Glenn (1968) stated that "...BUN retention is not highly specific nor highly sensitive as an indicator of the very mild states of renal dysfunction." It can be concluded, in the light of the above statements, that a BUN retention test may not be sensitive enough to detect very mild changes in the kidney. In this study, the dogs were housed in cages, did not work and were generally inactive. One might expect results to differ from those found among working military dogs because kidney damage becomes more pronounced by increased amount of work.

BUN and creatinine values were determined with the hope of detecting damage done by this organism directly or indirectly to the kidneys which in turn possibly could provide clues to bone marrow

depression, the apparent cause of anemia in canine ehrlichiosis. Erythropoiesis is controlled by a hormone, erythropoietin which is considered to be produced in large measure by the kidneys (Schalm, 1965 and Krantz and Jacobson, 1970). There are, however, no simple and accurate tests to determine erythropoietin levels and therefore it is common to measure it indirectly from kidney damage which is determined by creatinine values and BUN retention tests. In view of our results which reflect little or no kidney damage, the cause of bone marrow depression cannot be determined and still remains unknown.

Experiment 2. Ehrlichia canis, 1970 Oklahoma Isolate

Occurrence

Ewing, et al., (1971) reported for the first time the presence of Ehrlichia canis in Arkansas. The affected dog had traveled to several southcentral states and had become ill in March, 1970. The organism was found primarily in neutrophils rather than in lymphocytes and monocytes, and produced a milder form of canine ehrlichiosis than did the 1962 Oklahoma isolate (Ewing, et al., 1971). Earlier, Stannard, et al., (1969) and Gribble (1969) had reported ehrlichiosis from horses in California and stated that the causative agent was found primarily in neutrophils and eosinophils.

In July, 1970, the author diagnosed ehrlichiosis in a German Shepherd bitch admitted to the Small Animal Clinic at Oklahoma State University. The organism was similar to the Arkansas strain isolated by Ewing, et al., (1971) in that typical morulae were observed in neutrophils and eosinophils. Other dogs were experimentally infected

and morulae were never observed to parasitize lymphocytes or monocytes. This is the first report of the occurrence of a neutrophilic strain of E. canis in Oklahoma. Based on morphology, incubation period, and pathogenicity, as well as host cell affected, the organism appears to be similar to that reported by Ewing, et al., (1971) from Arkansas. No definite statement concerning taxonomic affinity can be made, of course, in the absence of serologic data and other information. Likewise, it is not known what relationship the 1970 Oklahoma isolate of E. canis may have with the Ehrlichia sp. observed by Stannard, et al., (1969) and Gribble (1969) in neutrophils and eosinophils of horses in California. Gribble (1969) found that sheep, goats and dogs were susceptible to infection, but cattle were not. The clinical signs attendant with infection in the susceptible species were mild or absent. In this instance at least, an Ehrlichia sp. with affinity for neutrophils was not pathogenic to dogs. Experiments should be conducted to determine whether the 1970 Oklahoma isolate of E. canis will produce disease in horses.

Incubation Period and Parasitemia

Ewing, et al., (1971) in their experimental infections of two dogs with the 1970 Arkansas strain of E. canis observed a rise in body temperature on the 16th day postexposure. The morulae of E. canis were found in the peripheral blood neutrophils beginning 20 and 21 days after exposure and persisted for approximately one week, and then disappeared.

In the present study 4 littermate pups (#1,5,6 and 8) were exposed to E. canis, 1970 Oklahoma isolate, by injection of 5 ml. of infectious

blood from a reservoir animal; and 2 additional littermate pups (#7 and 10) served as uninoculated controls. The body temperature pattern and parasitemia are shown in Figure 4 for one principal and a littermate control. Responses in the remaining principals and controls were almost identical and, therefore, graphs are not included.

The average incubation period in the four principals was 22.2 days and ranged from 20 to 24 days (Table II). Typical morulae were seen only in neutrophils and eosinophils (Figures 32, 33 and 34) of the peripheral blood where they persisted from 7 to 14 days (Table II) after their first appearance. These findings agree closely with observations made by Ewing, et al., (1971) on the Arkansas neutrophilic isolate. Both neutrophilic isolates remain in the peripheral blood for shorter periods than does the 1962 Oklahoma isolate which, in this study, persisted for as long as 40 days after their first appearance (Figure 3 and Table I). It is perhaps presumptuous to state that the mild strain of the organism is ablated from the peripheral blood at an early stage. Certainly relapses did not occur with the 1970 Oklahoma isolate within the period of this study, whereas all pups infected with the 1962 Oklahoma isolate did suffer relapse.

Long term persistence of E. canis (1970 Oklahoma isolate) in the host was not studied in these experiments; and, therefore, it is not possible to conclude the maximum period of persistence of this organism in dogs. However, it was found that one pup which was studied for 6 months after exposure was still infective and the organism could be transferred to susceptible dogs by intravenous inoculation of blood. It is not known in what tissues or organs the resistant phase of E. canis occurs. Jadin, et al., (1968, cited

by Ewing 1969) suggested that the closely related "...Rickettsia prowazeki has a resistant stage which persists in endothelial cells and that relapses occur when immunity is reduced." Ewing (1969) has demonstrated that the 1962 Oklahoma isolate of E. canis persists in convalescent animals for a period of 29 months, but conclusive evidence of where the organism resides has not been presented.

TABLE II

INCUBATION PERIOD AND PERSISTENCE OF EHRLICHIA CANIS (1970 OKLAHOMA ISOLATE) IN PERIPHERAL BLOOD OF FOUR PRINCIPALS ON DAYS AFTER EXPOSURE TO INFECTIOUS BLOOD

Identification No. of Pups	Incubation Period in Days	Persistence of E. canis Morulae on Days Postexposure
1	24	24,25,26,27,28,29,30,31
5	23	23,24,25,26,27,28,29,30
6	20	20,21,22,23,24,25,27,28,29,31
8	22	22,23,24,25,26,27,28,30,31

Clinical Signs

Clinical signs attendant with infection produced by the 1970 Oklahoma isolate in the four principals were few and quite mild. A slight rise of body temperature (Figure 4) occurred, on an average 21.5 days (range 18 to 24 days) postexposure. The duration of the febrile period varied from 7 to 14 days and roughly corresponded with the presence of morulae in the peripheral blood. The characteristic pattern of temperature fluctuations on alternate days produced by

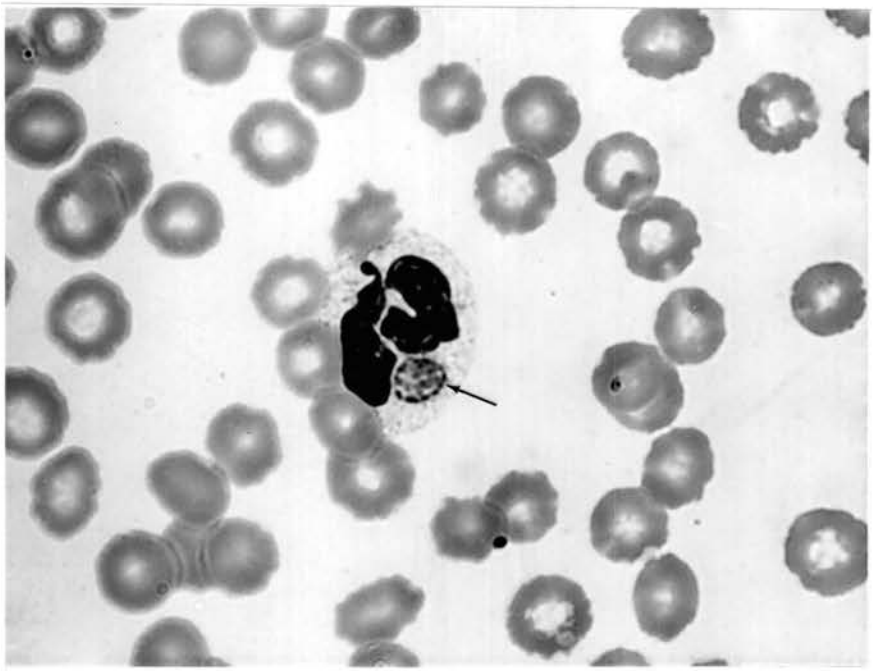

Figure 32. Neutrophil containing morula of Ehrlichia canis, 1970 Oklahoma isolate

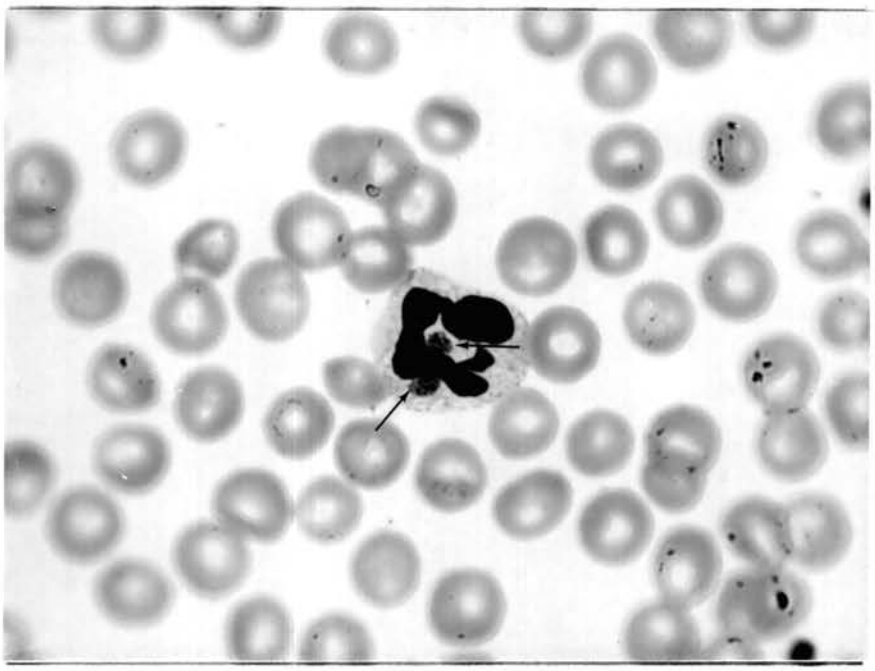

Figure 33. Neutrophil containing two morulae of Ehrlichia canis, 1970 Oklahoma isolate

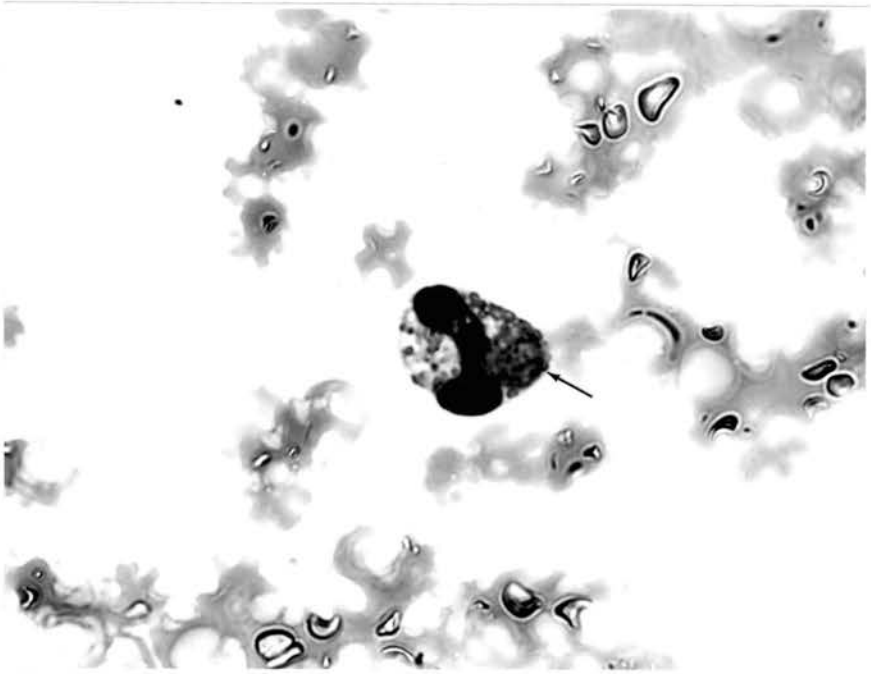

Figure 34. Eosinophil containing morula of Ehrlichia canis, 1970 Oklahoma isolate

the 1962 Oklahoma isolate did not occur in pups infected with this strain. The only other signs detected in these pups were bilateral, mucopurulent ocular discharge and, in some instances, serous nasal discharge. These discharges were first noticed soon after the febrile response began.

Hematology

From the foregoing discussion it is obvious that, based on currently available data, the 1970 Oklahoma isolate of E. canis produces a very mild disease as compared to that caused by the 1962 Oklahoma isolate, which is, in turn, milder than the disease produced by the strain or strains isolated in Asia. Such a conclusion is supported by hematological data gathered in this experiment. These data are presented in Figures 7,10,13,16,19 and 22. The lines on the graphs represent moving three-point averages of the mean values of principals and of controls. A very slight drop in the packed cell volume, hemoglobin value, and total erythrocyte count (Figures 7, 10 and 13) occurred in the principals but not in the littermate controls. These reduced PCV, hemoglobin values and erythrocyte counts lie within the normal range described for dogs by Schalm (1965). The 1970 Oklahoma isolate, therefore, affects the erythron adversely but not to a point to produce recognizable anemia.

The reticulocyte response in this litter of dogs can be discerned from Figure 16. It is difficult to evaluate the response because counts were high in the beginning of the experiment as a result of slight blood-loss anemia due to ancylostomiasis. The pups were treated with an anthelmintic in the pre-exposure study period, and the reti-

culocyte counts dropped quickly to within normal ranges. Reticulocyte counts of the principals were essentially identical to those of the littermate controls, indicating that the hematopoietic capabilities of the host were not impaired by the infection. It is evident that the 1970 Oklahoma isolate of E. canis behaves quite differently from the 1962 Oklahoma isolate; the latter has a definite effect on hematopoiesis, i.e., bone marrow depression for an extended period.

Total leukocyte count of dogs in this study can be seen in Figure 19. The count of the principals was slightly lower than that of littermate controls but remained within normal ranges for dogs of their age throughout the study. Absolute values of neutrophils decreased transiently in all four principals but not in the controls. This decrease roughly corresponded to the parasitemia (Figure 4 and Table II). Three principals (#5,6 and 8) experienced neutropenia beginning 27 days postexposure, and it persisted until 34 days after exposure. At this point neutrophils increased in the principals, reaching the lower limits of normal for dogs of that age but still were lower than the littermate controls. These findings agree with those of Gribble (1969) who observed neutropenia in horses infected with Ehrlichia. The mechanism governing this transient neutropenia is not known.

Data on thrombocyte counts are presented in Figure 22. The thrombocyte count was higher in both controls (#7 and 10) than in any principal during the pre-exposure period, a bias which could not be avoided because principals were randomly selected from the litter. During the postexposure period, the thrombocyte counts increased in both controls but stayed within the normal ranges for dogs stated

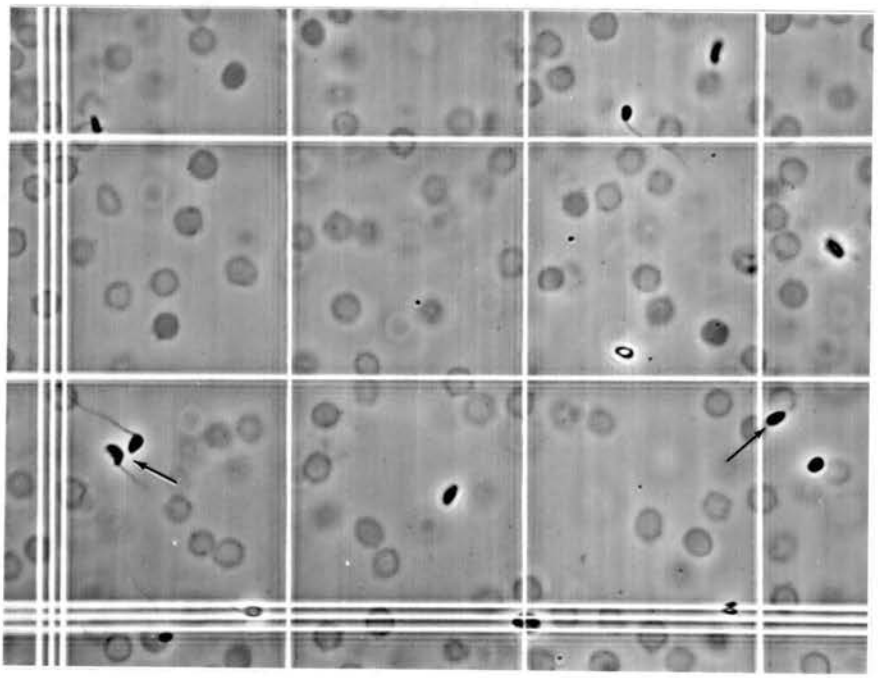

Figure 35. Thrombocytes from a pup infected with Ehrlichia canis, 1970 Oklahoma isolate. Notice only slight decrease in number and no effect on size of thrombocytes. The photomicrograph was made twenty-seven days after exposure.

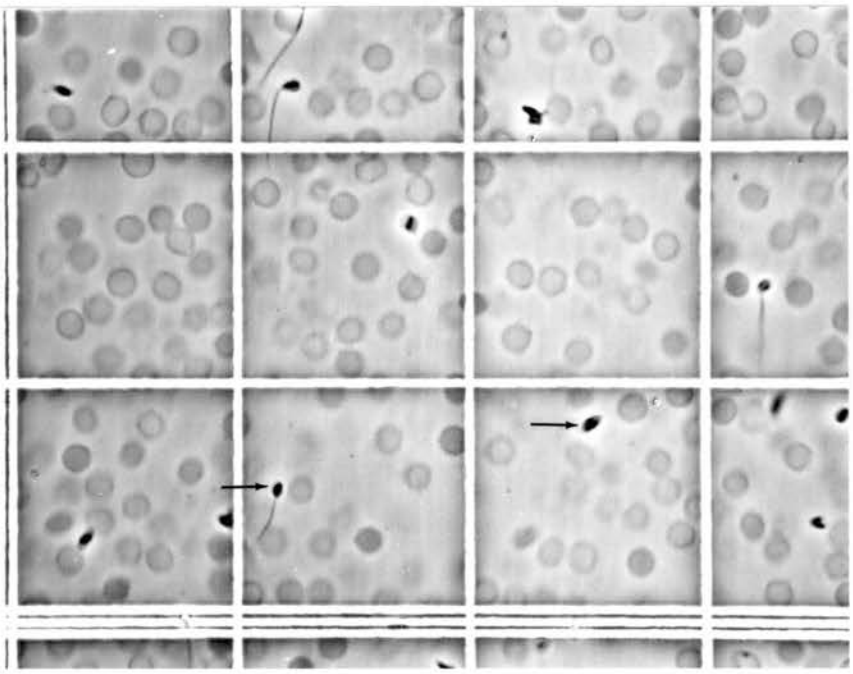

Figure 36. Thrombocytes from a littermate control twenty-seven days after exposure of principals. Compare the number of thrombocytes with those in Figure 35.

by Schalm (1965). At the same time, thrombocyte counts decreased in the 4 principals and the lowest level was reached 29 to 31 days after exposure. The values then gradually returned to the pre-exposure level. In no case did the thrombocyte counts of either principals or controls approach the sub-normal range stated by Schalm (1965). Gribble (1969), in his studies of equine ehrlichiosis, reported that thrombocytopenia occurred on days 4 through 12 postexposure and that less than 50,000 thrombocytes/cmm were a frequent observation. In the present study thrombocyte numbers were not as drastically decreased as were those of horses studied by Gribble (1969). It is difficult to evaluate the difference in effect of these two strains on thrombocytes because two different species of hosts are involved, and the taxonomic relationship between the two isolates of Ehrlichia is not known.

No increase in size of thrombocytes occurred in the principals in Experiment 2 (Figures 35 and 36). This is in sharp contrast to Experiment 1 in which there was a definite increase in the size of thrombocytes in pups infected with the 1962 Oklahoma isolate.

Chemical Studies

Certain chemical studies originally planned for Experiment 2, viz., creatinine, total and direct bilirubin and prothrombin time were not conducted. These determinations were deleted because results from Experiment 1 and other preliminary work made it clear that the information would not contribute to understanding the disease. Blood urea nitrogen, serum glutamic pyruvic transaminase and serum glutamic oxalacetic transaminase were determined throughout the experiment, however.

Liver Functions. SGP-T and SGO-T values are given in Figures 27 and 30. The principals had slightly higher values than did their littermate controls, but both principals and controls remained near the lower end of normal ranges described by Cornelius and Kaneko (1963). Based on these data it can be concluded that the 1970 Oklahoma isolate of E. canis does not have a detectable effect on either the liver or kidneys. This is in contrast to the 1962 Oklahoma isolate which, as seen in Experiment 1, produces slight liver damage.

Kidney Functions. There was no apparent difference in the values of BUN between the principals and their littermate controls. Therefore, graphs depicting BUN values are omitted from this dissertation.

Experiment 3. Ehrlichia canis, 1962 and 1970 Oklahoma Isolates, Separately in Littermate Pups.

Since the pups selected for Experiments 1 and 2 belonged to two different litters, a third experiment was conducted to compare the effects of both the 1962 and the 1970 Oklahoma isolates in pups from the same litter. This was done in an effort to eliminate variables, especially those of breed. Three pups (#21,22 and 24) were subjected to the 1962 Oklahoma isolate and an equal number (#27,28 and 29) to the 1970 Oklahoma isolate of Ehrlichia canis. One pup (#30) served as uninoculated control. Before the results are discussed, it is appropriate to state that all 7 pups were suffering from ancylostomiasis and were very anemic at the time they were obtained. It can be seen from the pre-exposure studies presented in Figures 8, 11 and 14 that their packed cell volume, hemoglobin values and total

erythrocyte counts were below normal ranges. One pup (#29), which later served as uninoculated control, had a PCV value of 18.5% when first examined. The reticulocyte count was also very high in all 7 pups during the pre-exposure studies. They were treated with disophenol,[1] injected subcutaneously, and with an aqueous iron solution,[2] administered orally. The response was excellent. At the time of exposure they were still slightly anemic but were gaining quickly. They were exposed at 8 weeks of age in order to approximate as closely as possible the two previous experiments.

Incubation Period and Parasitemia

Typical morulae were observed in lymphocytes of the peripheral blood in the three principals (#21,22 and 24) exposed to the 1962 Oklahoma isolate of E. canis after an average incubation period of 15 days, range 13 to 17 days (Table III). The pattern of appearance and persistence of morulae in the lymphocytes was essentially similar in all three principals. Morulae persisted in the peripheral blood for 10 to 12 days after their first appearance and then were not observed for a period ranging from 5 to 11 days after which they reappeared and persisted for 43 to 52 days. The absence of morulae in the peripheral blood corresponded with an interim period during which body temperature returned to normal (Figure 5). The temperature subsequently rose simultaneously with reappearance of morulae in the peripheral blood. The findings in Experiment 3 generally support the

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[1] D.N.P., Parenteral 4.5%; American Cyanamid Company; Princeton, N.J.

[2] Ferro Drops; Parke, Davis and Co.; Detroit, Michigan

results obtained in Experiment 1 except that they differed slightly in persistence of morulae in peripheral blood. In Experiment 1 morulae did not disappear from the peripheral blood soon after their first occurrence; in one instance they persisted for 40 days after their initial appearance (Table I and Figure 3). In addition to typical morulae large homogeneous bodies, presumably initial bodies, were observed in peripheral monocytes.

One interesting observation which has not been reported previously is the occurrence of typical E. canis morulae (1962 Oklahoma isolate) in the nucleus of peripheral lymphocytes. Morulae usually occur intracytoplasmically, but on a few occasions intranuclear inclusions were seen (Figure 37).

The disease produced by the 1970 Oklahoma isolate of E. canis had a slightly longer incubation period than did that produced by the 1962 Oklahoma isolate. The typical morulae of the 1970 Oklahoma isolate were observed in the peripheral blood neutrophils and eosinophils after an average incubation period of 20.3 days, range 20 to 21 days (Table III). These morulae persisted for a very short period, ranging from 5 to 10 days. Relapse did not occur in dogs infected with the 1970 Oklahoma isolate, and morulae were never found again in any of the pups even though they were checked daily until the experiment was terminated, a period ranging from 43 to 73 days postexposure. The findings in Experiment 3, then, support those of Experiment 2 as well as those of Experiment 1.

TABLE III

INCUBATION PERIOD AND PERSISTENCE OF BOTH ISOLATES OF *EHRLICHIA CANIS* IN PERIPHERAL BLOOD OF SIX PRINCIPALS ON DAYS AFTER EXPOSURE TO INFECTIOUS BLOOD

Identification No. of Pups	Incubation Period in Days	Persistence of *E. canis* Morulae on Days After Exposure
39^N	20	20,21,22,23,24,25,26,27,30
27^N	20	20,21,22,23,24,25
28^N	21	21,22,23,24,25,26,27,28
22^L	13	13,14,15,16,17,18,19,20,21,23, 25,31,32,33,34,35,36,37,38,40, 42,43
29^L	15	15,16,17,19,20,21,22,23,24,25, 32,35,36,37,38,47
21^L	17	17,19,20,21,23,24,26,27,37,38, 39,41,42,43

^{N}Pup infected with 1970 Oklahoma isolate

^{L}Pup infected with 1962 Oklahoma isolate

Clinical Signs

The first sign of infection with both strains of *E. canis* is rise in body temperature. The febrile period, in both cases, corresponds roughly with the presence of morulae in the peripheral blood (Figure 5). The body temperature of pups exposed to the 1962 Oklahoma isolate in this experiment never rose above 105°F and remained below 104°F in most cases; exacerbations and remissions occurred but were not as marked as those in Experiment 1 (Figure 3). Among those exposed to the 1970 Oklahoma isolate, the highest

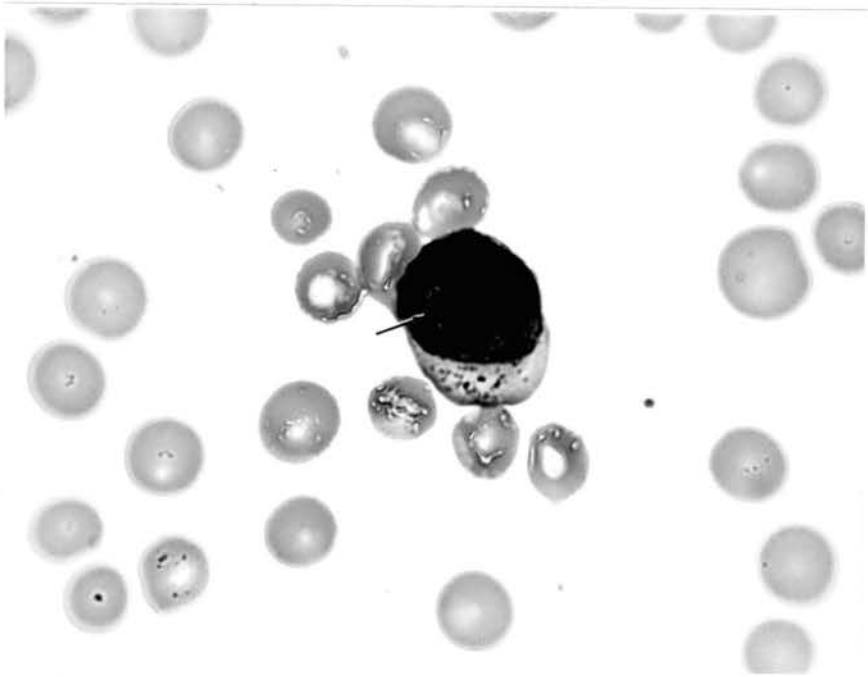

Figure 37. Morula of Ehrlichia canis, 1962 Oklahoma isolate, apparently within nucleus of lymphocyte. Morulae are normally intracytoplasmic.

temperature observed was 102.8°F and this was seen in one pup (#39) 25 days after exposure; these results are quite similar to those obtained in Experiment 2 (Figure 4). Other clinical signs were similar in type and intensity to those described in Experiments 1 and 2. The disease produced by the 1962 Oklahoma isolate was, however, less severe in pups in the third experiment than in those in Experiment 1, and none of the infected pups died.

It has been stated in the foregoing discussion that the 1970 Oklahoma isolate produces a milder form of ehrlichiosis than does the 1962 Oklahoma isolate, but it has similarities to the 1970 Arkansas isolate reported by Ewing, et al., (1971). Although the dogs exposed to the 1962 Oklahoma isolate in this experiment were not affected as severely as those in the Experiment 1, the general pattern of the disease was similar. None of the pups died in Experiment 3, whereas three pups (#72,74 and 77) died in Experiment 1. It is not possible to state why the dogs reacted differently, but differences in breed susceptibility to canine ehrlichiosis are well documented in the literature (Huxsoll, et al., 1970 and Seamer and Snape, 1970). Seamer and Snape (1970) in their study of tropical canine pancytopenia, apparently a severe type of ehrlichiosis, found that clinical signs were minimal in five experimentally infected mongrels while Beagles were more severely affected. Huxsoll, et al., (1970) stated that "...epistaxis and other forms of hemorrhage associated with thrombocytopenia have occurred in experimentally infected German Shepherds as early as 10 days and up to 120 days post-inoculation. Clinical signs of hemorrhage have not been observed in laboratory Beagles experimentally infected with agents

of TCP and relapses are less common in Beagles." One possible explanation, therefore, for the disparity in results of Experiment 1 and Experiment 2 is breed susceptibility. The pups in Experiment 3 were mongrels of Collie and English Setter ancestry whereas those in Experiment 1 were a mixture of Collie and German Shepherd. The latter breed, of course, has been cited as the most severely affected by canine ehrlichiosis (Huxsoll, et al., 1970).

Hematology

Figures 8,11,14,17,20 and 23 depict the hematological changes which developed in six principals (#21,22,27,28,29 and 39) suffering from experimentally induced ehrlichiosis and their unexposed littermate control (#30).

The PCV, hemoglobin values, erythrocyte and reticulocyte counts can be interpreted to mean that pups infected with the 1962 Oklahoma isolate apparently were unable to replace erythrocytes rapidly enough to keep pace with the need, and anemia developed. The development of anemia corresponds well with the persistence of morulae in the peripheral blood lymphocytes. The PCV, hemoglobin values and total erythrocyte counts decreased soon after exposure and declined to the lowest level by 17 to 22 days after exposure. All values then increased concurrently with the disappearance of fever and parasitemia, only to decrease again after a period roughly corresponding to the recurrence of fever and of morulae in the peripheral blood. The morulae persisted to the end of this study period which was terminated earlier (43 days postexposure) than was Experiment 1 because the pups in the first experiment were studied long enough

(67 to 92 days postexposure) to observe convalesence. It was thought that it would not be worthwhile to prolong the studies in Experiment 3 since studies on the recovery phase were made in the first Experiment.

In littermate pups exposed to the 1970 Oklahoma isolate, PCV, hemoglobin values and total erythrocytes decreased slightly when compared with the control, but the decrease was much less marked than that observed in pups infected with the 1962 Oklahoma isolate. Severe anemia did not develop in any of the pups infected with the 1970 Oklahoma isolate. PCV, hemoglobin values and erythrocyte counts reached their lowest level 17 to 31 days postexposure and then increased quickly, reaching the level of the littermate control by 24 to 34 days postexposure.

Reticulocyte counts (Figure 17) in the pups infected with the 1962 Oklahoma isolate decreased quickly, but this decrease was never as drastic as seen in Experiment 1. Anemia accompanied by a reticulocyte count which was within the normal range indicated that the hematopoietic capabilities of the animal were depressed. This finding was quite different from the results obtained in pups infected with the 1970 Oklahoma isolate; in this case PCV, hemoglobin values and erythrocyte counts dropped only slightly when compared to the control. The reticulocyte counts remained even and at a slightly higher level than the control throughout the experiment. This clearly indicates that the hematopoietic capabilities of the host are not depressed by the 1970 Oklahoma isolate at any period of the infection. It has been stated in the foregoing discussion that bone marrow depression is usually considered the major factor in the production of anemia in canine ehrlichiosis. It can be concluded from our findings of

(1) severe anemia accompanied by normal rather than elevated reticulocyte counts in pups exposed to E. canis, 1962 Oklahoma isolate and (2) absence of anemia but high reticulocyte counts in those exposed to the 1970 Oklahoma isolate that anemia results from bone marrow depression in one instance and fails to develop in the other. The difference in the response of bone marrow to the organism would appear to be a function of variations in the strain of E. canis studied.

The total leukocyte count (Figure 20) of pups exposed to the 1962 Oklahoma isolate decreased slightly for a short period of 3 to 7 days beginning 10 to 20 days after exposure and then increased. This increase corresponded to the disappearance of morulae from the peripheral blood. A second decline occurred 34 to 38 days post-exposure and persisted to the end of the experiment; this second occurrence of leukopenia was accompanied by recurrence of parasitemia (Figures 5 and 20). In contrast, pups exposed to the 1970 Oklahoma isolate did not suffer marked leukopenia, and the leukocyte counts were almost identical to those of the control. Lymphopenia was not marked in any of the pups infected with the 1962 Oklahoma isolate at the onset of illness but was observed during the relapse which occurred 31 to 38 days postexposure (Figure 5). The relapse was judged by the presence of morulae in the peripheral lymphocytes and by the febrile response. A slight neutropenia was observed in one pup (#78) infected with the 1970 Oklahoma isolate in this experiment; all pups infected with the same strain in Experiment 2 experienced neutropenia. This slight difference in response of pups to the same isolate of E. canis in Experiments 2 and 3 may be a reflection

of difference in breed susceptibility. This cannot be proved, however, on the basis of the present data.

Results of thrombocyte counts in the three pups infected with the 1962 Oklahoma isolate are shown in Figure 23 and agree closely with the findings of Experiment 1. There was a sharp decline in the thrombocyte count 17 to 20 days after exposure and in one instance was only 32,000/cmm. Thrombocytes then increased slightly for a short period only to decline again 31 to 38 days postexposure (Figure 23). This transient increase in thrombocytes roughly corresponded with the period during which morulae disappeared from the peripheral blood. The lowest thrombocyte count observed was 25,000 and occurred 24 days after exposure. Counts never returned to normal, even by the end of the experiment 73 days after exposure. Two pups from each group were euthanatized 43 days postexposure while studies on one pup from each group were extended for 73 days post-exposure. Thrombocytopenia persisted to the end of the experiment in the pup infected with the 1962 Oklahoma isolate.

A definite increase in the size of the majority of thrombocytes was observed in pups infected with the 1962 Oklahoma isolate. Some of the thrombocytes from principals were twice the size of those of the control (Figures 24 and 25). The thrombocytes of pups infected with the 1970 Oklahoma isolate remained unchanged and apparently were identical to those of their littermate control (Figures 35 and 36). The reason for and significance of enlargement of thrombocytes in pups infected with the 1962 Oklahoma isolate is not known.

The thrombocyte response in the three pups (#27,28 and 29)

infected with 1970 Oklahoma isolate was consistent and very mild (Figure 23). The thrombocyte counts decreased slightly when compared with the littermate control but remained within normal ranges for dogs of their age throughout the experiment; the counts returned to the level of the littermate control at the end of the experiment. These results contrast sharply with those obtained in dogs infected with the 1962 Oklahoma isolate; in the latter instance, as stated earlier, severe thrombocytopenia developed soon after exposure and persisted to the end of the experiment. Presumably the severe thrombocytopenia caused by the 1962 Oklahoma isolate results from bone marrow depression.

Chemical Studies

Creatinine, and total and direct bilirubin values were not determined in this experiment. Determination of prothrombin time was discontinued in the middle of the experiment because of reasons mentioned elsewhere. Determinations of blood urea nitrogen (BUN), serum glutamic pyruvic transaminase (SGP-T) and serum glutamic oxalacetic transaminase (SGO-T) were made throughout this experiment, however.

Liver Functions. SGP-T and SGO-T values are presented in Figures 28 and 31. SGP-T values in the three pups infected with the 1962 Oklahoma isolate reached a maximum level ranging from 36 to 39 Sigma Frankel units after a period ranging from 22 to 24 days postexposure. SGO-T values were also elevated during this period. These SGO-T and SGP-T values, when compared with the control, are slightly elevated; but they are within normal ranges described for

dogs by Cornelius and Kaneko (1963). The response in the pups infected with the 1962 Oklahoma isolate in Experiment 3 was essentially the same as that seen in Experiment 1 but was less marked. SGP-T and SGO-T values in three pups infected with the 1970 Oklahoma isolate were always within the lower limit of normal indicating that this organism does not damage the liver sufficiently to be detectable by techniques presently available. This finding is in contrast, of course, to the result from dogs infected with the 1962 Oklahoma isolate.

From the present data and foregoing discussion it can be concluded that the disease produced by both these isolates of E. canis are similar and differ from each other largely in degree of pathogenicity. The 1970 Oklahoma isolate produced a mild disorder in both naturally infected animals and experimentally exposed dogs as compared to the 1962 Oklahoma isolate which produced an illness characterized by severe anemia, leukopenia, thrombocytopenia and slight liver necrosis.

Kidney Functions. Graphs showing BUN values for Experiment 3 are omitted from this dissertation since no change was observed in BUN values of six principals in comparison with the littermate control. It can be stated, therefore, that neither isolate affects the kidneys sufficiently to be detected by the methods used in these experiments.

CHAPTER V

SUMMARY AND CONCLUSIONS

The occurrence of a mildly pathogenic strain of Ehrlichia canis is reported from Oklahoma and is referred to as the 1970 Oklahoma isolate. A second strain of E. canis, referred to as 1962 Oklahoma isolate, was confirmed to be very pathogenic. The incubation period of the disease produced by the 1962 Oklahoma isolate was shorter than that of the 1970 Oklahoma isolate. The morula-type aggregates of the organisms in the 1962 Oklahoma isolate were observed in peripheral lymphocytes from 11 to 16 days after exposure to infectious blood and were detectable in the peripheral blood for as long as 40 days after their first appearance. Morulae of the 1970 Oklahoma isolate, on the other hand, occurred in peripheral neutrophils and eosinophils but never in lymphocytes; they were observed first 20 to 26 days after exposure and persisted in peripheral blood for a short period ranging from 5 to 14 days after their first appearance. Relapse, characterized by increase in body temperature and recurrence of parasitemia, occurred in pups infected with the 1962 Oklahoma isolate but not in those exposed to the 1970 Oklahoma isolate.

Clinical signs attendant with infection by the 1962 Oklahoma isolate were severe, and body temperature fluctuations were very characteristic showing exacerbations and remissions on alternate days and temperature reaching as high as 107°F. Clinical signs in pups

infected with the 1970 Oklahoma isolate were very mild; the characteristic pattern of temperature fluctuations on alternate days produced by the 1962 Oklahoma isolate was not observed in pups infected with this strain. The highest temperature observed was 102.8°F. The duration of the febrile period in pups infected with either isolate corresponded roughly with the presence of morulae in the peripheral blood. The morulae in the peripheral blood lymphocytes were few or absent during the remission period in pups infected with the 1962 Oklahoma isolate.

Based on hematological studies, i.e., packed cell volume (PCV) hemoglobin, erythrocyte counts and reticulocyte counts, it is concluded that pups infected with the 1962 Oklahoma isolate were unable to replace erythrocytes rapidly enough to keep pace with the need. Hematopoietic capabilities of the host were impaired and severe anemia developed. In contrast, values of PCV, hemoglobin, total erythrocytes and reticulocyte counts in pups infected with the 1970 Oklahoma isolate decreased only slightly when compared with controls, and severe anemia never developed. It was concluded that the 1970 Oklahoma isolate affects the erythron by an unknown mechanism but not to a point to produce recognizable anemia; apparently the hematopoietic capabilities of the host infected with this isolate were not seriously impaired.

Leukopenia and severe thrombocytopenia were observed in pups infected with the 1962 Oklahoma isolate. Although thrombocyte counts in principals infected with 1970 Oklahoma isolate were lower than those of littermate controls, they were within normal range and their size remained unchanged.

From organ function studies it is concluded that the 1962 Oklahoma isolate produces slight liver necrosis while the 1970 Oklahoma isolate does not affect this organ. Neither isolate affects the kidney adversely, at least not to an extenct that can be detected with techniques used in this study.

It is concluded that the diseases produced by these two isolates of E. canis differ from each other in degree of severity. The 1970 Oklahoma isolate produced a mild disorder in both naturally infected animals and experimentally exposed dogs as compared to the 1962 Oklahoma isolate which produces a severe illness characterized by high fever, anemia, leukopenia, thrombocytopenia, and slight liver necrosis. Anemia, leukopenia, thrombocytopenia and liver necrosis were not seen in pups infected with the 1970 Oklahoma isolate.

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