AN ABSTRACT OF THE THESIS OF

William Edward Noonan (Name) for the Doctor of Philosophy (Degree)

in Zoology (Major) presented on July 7, 1972 (Date)

Title: NEORICKETTSIA HELMINTHOECA IN CELL CULTURE

Abstract approved: Redacted for Privacy

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The etiologic agent of salmon poisoning disease was found to be Neorickettsia helminthoeca, although a second organism, the Elokomin fluke fever agent, may also be involved in other areas. Primary cultures of dog leucocytes were found to support the in vitro cultivation of Neorickettsia helminthoeca as were canine sarcoma 503 cells and mouse lymphoblasts MB III. Quantitative methods were not applied to in vitro studies of multiplication of the rickettsia because it failed to grow sufficiently in laboratory animals or in chicken embryo yolk sacs to do so.

By ultrastructural analysis it was learned that Neorickettsia helminthoeca was structurally similar to other rickettsiae. Rickettsial cells were seen as circular profiles or rod-shaped cells 0.5μ wide and up to 0.7μ long. They were bounded by a cell wall and an underlying cytoplasmic membrane. Each of the membranes was a trilayered structure and showed the unit membrane structure.

Koch's postulates were fulfilled as completely as possible for an organism that is an obligate intracellular parasite. Neorickettsia helminthoeca was found in all cases of salmon poisoning disease. The rickettsia was isolated and grown in culture. The isolated culture was found to reproduce the disease when inoculated into susceptible dogs. The rickettsia was observed in and recovered from the inoculated dog.

Neorickettsia helminthoeca in cell systems can be used as a model to further investigate the host-parasite relationship.

Neorickettsia helminthoeca in Cell Culture

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1973

APPROVED:

Redacted for Privacy

Professor of Zoology
in charge of major

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Date thesis is presented July 7, 1972

Typed by Cheryl E. Curb for William Edward Noonan

Parasitism may be regarded, not as a pathological manifestation but as a normal condition having its roots in the inter-dependence of all living organisms.

Theobald Smith

ACKNOWLEDGMENTS

I gratefully acknowledge the guidance and advice of Dr. Ivan Pratt under whose direction this study was made. I wish particularly to thank Dr. A. Owczarzak without whose essential advice and facilities for tissue culture the project could not have been carried out. I also acknowledge the assistance of the following individuals: Mr. Lyle Bogenhagen, Dr. Stuart Knapp, Dr. Donald Mattson and Dr. Paul Presidente of the Department of Veterinary Medicine, Oregon State University; Dr. K. Farrell, Department of Veterinary Medicine, Washington State University; Dr. James Akiyama, Department of Biological Sciences and Microbiology, San Jose State College; Mr. Kay Fernald, Dr. H.K. Phinney and Mr. Al Soeldner, Department of Botany, Oregon State University; Dr. S. Pilcher, Department of Microbiology, Oregon State University; Mr. Donald Morrison, Department of Zoology, Oregon State University; and Mr. Paul Vroman, Oregon State Game Commission.

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NEORICKETTSIA HELMINTHOECA IN CELL CULTURE

INTRODUCTION

Salmon poisoning, a disease of the Canidae, was indigenous to southwestern Washington, western Oregon and northwestern California before this region was settled by the pioneers.

Henry (1814) mentioned that dogs from Astoria were sent up to the Willamette for the winter to prevent their death, as living on raw salmon the previous summer and fall had caused many losses. Thornton (1849) further qualified this relationship by noting that dogs are very fond of raw salmon, but it is usually fatal to them. He considered only fresh salmon to be injurious and related that an opinion generally prevailed that a poison was contained in the blood of the salmon. This poison caused a "dull and moping" condition in dogs the second day after ingestion. This was followed on the fourth day by rejection of food except salmon which was eaten until expiration about the tenth day. Cooper and Suckley (1859) observed that blooded dogs introduced from the States became very ill after eating raw salmon although the native dogs of Oregon subsisted well upon it. Bonebrake (1925), having observed the disease in 1862, also reported this condition which was called "salmon sickness". It was, however, thought by some to be common dog distemper.

Nash (1882) explained that salmon returning to spawn were deadly poison to dogs and wolves. He did not have any faith in the remedies such as epsom salts, castor-oil, large doses of mustard, shot in quantities forced down the throat, calomel, aloes and blackberry-tea that were prescribed as antidotes for the poison. Since most mature dogs became "salmoned" and died he suggested immunity could be obtained by allowing puppies free run of salmon. Survivors having passed the ordeal were salmon-proof and lived to their full age. Preventive advice of keeping valuable dogs tied up was offered to newcomers into the coast country.

Pernot (1911) related that the most fatal disease of dogs in Oregon was commonly known as "salmon poisoning". This disease was commonly explained by the theory that salmon possess a poison which when eaten by dogs produced sickness and death. He reasoned since all diseases have a specific cause it was necessary to determine "the specific cause". He assumed that since the disease had an incubation period of nine days after ingestion of salmon that it must be bacterial in nature. Pernot isolated pure cultures of microorganisms from salmon, cultured them in bouillon and fed them separately to dogs. None of the cultures, either singly or mixed, gave symptoms of the disease. He reasoned that the disease must be caused by the multiplication of some living parasite during the incubation period. This led him to make a systematic study of the salmon and the blood of an infected dog.

He fed a dog one-quarter of a teaspoonful of the dark bloody substance taken from along the backbone of the inside of the salmon (kidney tissue) and produced salmon poisoning disease. He drew a fraction of a drop of blood from this dog and injected it subcutaneously into a healthy dog. After nine days the dog developed a typical case of salmon poisoning. The experiment was repeated using a guinea pig which did not show any signs of disease after 15 days. However, the disease was produced by feeding one inch of the lower intestine of an infected dog to a susceptible dog. These facts convinced Pernot that some living parasite was transferred from a diseased dog to a healthy one and that the parasite multiplying during the incubation period produced the disease and subsequent death. The disease signs did not occur upon challenge with infective salmonid kidney if the dog had been "salmoned" previously or if the salmon had been cooked. Upon close examination of the salmonid kidney numerous small white dots were found which Pernot thought were encysted amoebae.

Donham (1925) first linked a trematode with salmon poisoning disease. The trematode was found in the intestinal tract of dogs that had died after eating "sore-back" salmon. Cysts were found in the muscles of the salmon used in the experiment. Donham inferred that the cysts were intermediate forms of the mature fluke found in the intestine of affected dogs.

Wyatt (1925) attempted to isolate a substance from salmon which would cause the dogs to be poisoned. Alcoholic extracts were made from salmon flesh, evaporated and dissolved in ether. This ether soluble fraction, oily in nature, contained a substance which he thought caused illness in dogs. Wyatt's description of the illness, autopsy findings and blood counts suggest he was not dealing with salmon poisoning but rather with typical canine distemper.

Donham, Simms and Miller (1926) defined the geographical distribution of salmon poisoning disease. The disease was found to occur in western Oregon, northwestern California and southwestern Washington, but not in British Columbia nor Alaska although salmon and trout were plentiful in these areas. They found the cause of the disease to be a small undescribed intestinal trematode, the mature form of which occurred in the intestines of affected mammals. A cystic form was found to be very numerous in the kidney and muscles of infected fish and somewhat less numerous in the gills and liver. Ward and Mueller (1926) observed cysts in the eyes, optic nerves, brains as well as in the kidneys, muscles, gills, livers, and walls of the digestive tubes. They suggested the name Distomulum oregonensis based only on the metacercariae. The metacercaria was demonstrated to develop into the mature parasite in seven to ten days by feeding fish containing the cysts to dogs which were necropsied at various time intervals. Cooking or prolonged freezing of the fish killed the

metacercariae.

The life cycle of the parasite was not completely known other than that the cyst developed into the mature fluke found in the dog and then produced eggs which passed out in the fecal material of the dog. Donham et al. (1926) discussed the symptoms of the disease and noted that signs did not appear until seven to ten days after ingestion of infected fish. The incubation period corresponded to the time required for the fluke to reach maturity. The temperature of the dog increased to 105°-107°F. and was accompanied by depression, anorexia, thirst and in some cases by a discharge from the eyes. After twenty-four to forty-eight hours the temperature decreased and the animal became progressively weaker, emaciated and died about six to eight days after onset of symptoms.

The trematode was identified by Chapin (1926) as a new species belonging to a new genus of the family Heterophyidae and was described as Nanophyes salmincola, but he (1928) later noted that the name Nanophyes was preoccupied and changed it to Nanophyetus making the name Nanophyetus salmincola (Chapin).

Donham and Simms (1927) established through experimental infections that the coyote, Canis latrans, was also susceptible to salmon poison disease.

Donham (1928) found that natural immunity to salmon poisoning does not occur. Four litters totaling twenty-one puppies, fifteen of

which were from an immune female, were used for experimental infections. All twenty-one of the puppies were susceptible. There was no experimental evidence that sex, age, breed, physical condition, care or rations affected susceptibility. Acquired immunity in the form of a definite, permanent immunity was found to be present in all dogs that recovered from the disease.

Donham (1928) aseptically injected defibrinated blood from an immune dog into three susceptible dogs in an effort to transfer immunity. The blood serum was given to two of the dogs before symptoms developed and to one after the symptoms developed. All of the dogs died.

Attempts were made to demonstrate the presence of endotoxins or exotoxins produced by the mature flukes. Three dogs were given intraperitoneal injections of "ground up" mature flukes. All of the dogs died. One dog which had received a subcutaneous injection of the material lived.

He attempted to experimentally establish the minimum number of metacercariae required to produce "poisoning" in susceptible dogs. The results were incomplete although it was established that typical salmon poisoning could be produced by one hundred cysts in four out of ten dogs tested.

Donham (1925, 1928) tried to determine if a snail acted as the intermediate host of Nanophyetus salmincola. Oxytrema silicula

(=Goniobasis plicifera var silicula) was found to be present in almost every stream throughout the geographical distribution of the disease. He established that fish under controlled conditions became parasitized when exposed to infected snails. Donham (1928) concluded that salmon poisoning was associated with the intestinal trematode, although he considered the symptoms and course of the disease indicative of an infectious agent rather than the result of parasitism by the trematode. Since dogs that had recovered had acquired a definite active permanent immunity, an infectious etiology was probable. He further reasoned that the trematode, Nanophyetus salmincola, merely provided a mode of entrance for this agent.

Donham, Simms and Shaw (1928) studied the longevity of metacercarial cysts stored in dead fish at 35°F. and found them to be viable when fed to dogs at 74 days but not at 94 days. The cysts when spread in a thin layer and dried at room temperature for five days did not produce disease when fed to susceptible dogs. The disease was also produced by intraperitoneal injection of washed mature flukes suspended in water.

Sinitsin (1931) described the cercaria of Nanophyetus salmincola which developed in Oxytrema silicula and found that feeding experiments disclosed that guinea pigs were susceptible to trematode infection but although they had thousands of worms in their intestine, no sign of salmon poisoning appeared.

Transmission of the etiological agent of salmon poisoning disease and immunization against it were studied by Simms, McCapes and Muth (1932). They found the disease was not transmitted by kennel exposure, although injection of defibrinated or whole blood (0.1 ml-5.0 ml) intraperitoneally or subcutaneously from sick dogs consistently produced the disease. In one instance the disease was passed through sixteen successive dogs by this method before the experiment was terminated. Attempts to pass the agent through Mandler (medium porocity) and Seitz filters have been negative as was feeding ten c.c. of "virulent blood" (blood obtained from an infected dog) to a susceptible dog. Intraperitoneal injection of metacercariae produced typical disease signs although injection of rediae and cercariae in 85 percent saline did not. The blood of a salmon did not transmit the infection nor did the blood of a raccoon, Procyon lotor, which had been fed metacercariae. Infection of virulent blood into a guinea pig, white rat, rabbit and raccoon did not produce typical signs of the disease.

Simms et al. (1932) established that 16 of 20 dogs could be immunized against the disease through simultaneous injection of virulent blood (0.1 ml-2.0 ml) and hyperimmune serum (16 ml-40 ml) which was produced by injecting virulent blood into the peritoneal cavities of immune dogs.

Simms and Muth (1934) elaborated on the characteristics of the causal agent by conducting further experiments on transmission of the disease with virulent material which had been treated in various ways. Serum collected from experimentally infected dogs, citrated and centrifuged at 2,000 rpm for 30 minutes did not produce the disease when injected into susceptible dogs. Citrated blood from an infected dog was washed free of serum through repeated centrifugation and washing with 0.85 percent sodium chloride solution. This process was repeated 16 times at which time an equal part of sodium chloride solution was added to the cells. One ml of the mixture was injected into a susceptible dog which developed typical signs of the disease. Washed erythrocytes, free of white blood cells, did not produce the disease in three of four dogs tested. Injection of dried blood, phenolized (0.5 percent) blood, or blood "exposed in vitro by hyperimmune blood" failed to produce the disease. Virulent blood heated to one hundred forty five degrees for five minutes did not produce the disease; however, blood heated to one hundred and twenty-seven degrees Fahrenheit for two minutes produced typical symptoms. The effect of freezing on the infective agent is unclear. Blood stored two days at forty degrees Fahrenheit did not lose its infectivity, although it did when stored for thirty-six days. Injection of ground flukes obtained from the intestines of dogs which had died of salmon poisoning regularly produced disease in susceptible dogs. Flukes from immune dogs were

also infective. Flukes were recovered from a guinea pig which had been fed 500 metacercariae. These flukes were ground in saline and injected into susceptible dogs without eliciting symptoms of the disease. A susceptible female dog was mated to a male which was showing symptoms of disease. The female developed typical symptoms and her blood proved virulent when injected into susceptible dogs. Young puppies from immune dams were found to be susceptible while those from hyperimmune ones were resistant.

Simms and Muth (1934) tried to immunize dogs with tissue vaccines made of spleen; kidney, liver, intestinal mucosa, lymph glands or citrated blood. The tissues were removed from the dogs on the ninth to thirteenth day after exposure to the disease and were ground, weighed and mixed with an equal weight of 0.85 percent sodium chloride. One percent of chloroform was added and the tissue was stored at forty degrees Fahrenheit for three days. Those vaccines containing considerable amounts of reticulo-endothelial tissues produced immunity.

The transmission experiments of Simms and Muth (1934) prompted them to speculate that the causal agent could exist only when it was associated with living cells especially those of the reticulo-endothelial system. The agent was destroyed by freezing, drying and heating and its absence from serum of virulent blood indicated it was not of the same nature as bacteria. The negative filtration experiments,

destruction by freezing, drying or addition of 0.5 percent phenol eliminated the viruses. These findings led them to conclude that the causal organism was not closely related to any known organism, it was logical to consider the rickettsia or some of the hemosporidia.

Coon et al. (1938) clarified further the nature of the causative agent by finding sulfanilamide to be effective in treatment of dogs. Improvement accompanied by a drop in temperature and return of appetite was noted within twenty-four to forty-eight hours after administration. This treatment was found to produce immunity to the disease, which was tested by Shaw and Howarth (1939), who challenged treated dogs with infective material without producing the disease.

Cordy and Gorham (1950) further elaborated the etiology and pathology of the disease in dogs and foxes. They aseptically removed the spleen and lymph nodes of a fox and suspended them in sterile skim milk with a semimicro Waring blender. This material was infective for dogs and foxes but not for mink. Broth suspensions were found to be infective for dogs and foxes but not for cats, mink, guinea pigs or hamsters. Broth suspensions of lymph nodes were used to innoculate three to five week-old mice intraperitoneally. Three serial passages were made by each of three routes. Intraperitoneally, by fox lymph node suspension in the first passage, ten percent broth suspension of pooled mouse spleens in the second and third passages, intranasally, using pooled mouse lungs in the second and third

passages, and intracerebrally using mouse brains for the latter passages. Gross lesions were not apparent in any of the mice. The brains, lungs and spleen of the third passage mice were used to prepare a 17 percent saline suspension which was infective to dogs and foxes.

Four dozen chicken eggs incubated six days were innoculated in the yolk sac with various dilutions of fox lymph node suspensions. Yolk sacs were resuspended in broth and used for further passages. No apparent growth was observed in any of the eggs and when yolk sac suspensions were injected into dogs illness did not result.

Cordy and Gorham (1950) found that macroscopic lesions were similar in both dogs and foxes but they were more severe in foxes. Changes in the lymphoid tissue appeared to be primary and were found in all cases. The principle gross and microscopic lesions were found to include hyperplasia of visceral and somatic lymph nodes, sometimes with hemorrhage or necrosis; variable hyperplasia of the spleen, intestinal lymphadenoid tissue and thymus; and hemorrhage of the gastro-intestinal tract and lungs. It was the histopathologic examination of these animals which led to the discovery of elementary bodies. In all of the foxes and dogs observed, small intracytoplasmic bodies were observed in the large reticulo-endothelial cells of lymph nodes, tonsils, spleen, intestinal lymph follicles and thymus. They were occasionally seen in macrophages of the lower lungs and blood and in parenterally infected animals they were found in serosal macrophages.

These bodies were coccoid or coccobacillary and of a uniform size of about three hundred millimicrons. The bodies were found to occur in the cytoplasm of reticulo-endothelial cells in compact plaques or loose groups, often nearly filling the cytoplasm.

Cordy and Gorham (1950) reasoned that since elementary bodies were always found in infected animals and never in uninfected controls that these bodies must be the etiologic agent of the disease. Bacteria could not be implicated since growth did not appear on standard media. They assigned the causative organism to the order Rickettsiales. Since an arthropod vector was not involved and erythrocytes were not parasitized the organism was placed in the family Chlamydozoaceae. Members of this family are effectively treated with sulfanilamide, but cannot be cultured in cell-free media.

Cordy and Gorham (1951) established that sulfonamides, penicillin, aureomycin and chloromycetin were highly effective in the treatment of experimental salmon poisoning in dogs.

Coles (1953) could not see any justification for separating the Chlamydozoaceae from the Rickettsiaceae on the single fact that none of the former had been shown to depend on arthropods for transmission. Philip, Hadlow and Hughes (1953) considered the unique transmission by a helminth as well as the reported differences from the typhus-like Rickettsia response to chemotherapeutic and antibiotic agent to justify giving the causative agent of salmon poisoning disease the new generic

and specific name, Neorickettsia helmintheca. They felt it appeared more correctly placed in the Rickettsiaceae than in the Chlamydozoceae although the vector characterization of the former family would have to be revised to include helminths. Philip, Hadlow and Hughes (1954) suggested that strict Latin derivation resulted in helminthoeca rather than helmintheca which was used in their original description.

Philip et al. (1954) were unable to infect chicken embryos using both blood and lymph node suspensions from dogs. Guinea pigs were found to carry the infection through six passages. First passage animals developed fevers of four to five days duration and had enlarged mesenteric lymph nodes. These signs became less apparent with continued passage. The sixth passage guinea pigs remained afebrile. A similar history of retrogressive node involvement occurred in blind passage of lymph node suspensions in hamsters and mice. Mice which had been injected thirty-nine days previously with dog lymph node suspensions were found to cause a febrile reaction when their pooled lymph nodes were injected into a susceptible dog.

Growth was not found to occur using infected lymph node suspensions in various bacteriological media. Cultivation attempts by Philip, Hadlow and Hughes (1954) at temperatures between 19 and 34°C in minced chicken embryos in Ringer's solution, on Zinsser-Plotz flasks, or on modified Dorset's egg medium were not successful. They suggested that tissue culture merited further investigation.

The extra-vertebrate phases of Nanophyetus salmincola were studied by Philip et al. (1954). They found that the lymph nodes from an experimentally infected dog that had recovered spontaneously were not infective. Approximately three hundred adult trematodes, however, from the recovered dog were infective to susceptible dogs. This observation supported the contention that the infection may be residual in trematodes while not demonstrable in node tissues of the recovered host. Trematodes from a symptomless, Florida raccoon fed fish harboring metacercariae were also infective to dogs.

Survival of Neorickettsia helminthoeca in suspensions of trematodes was found to be limited. Filtration attempts were invalidated since the agent was not even persistent in the unfiltered control through the two hours (at room temperature) required for the operation.

Fluke stages from dissected livers of Oregon snails were found to be infective when injected peritoneally into susceptible dogs. Eggs were not found to be infective when injected in a similar manner.

Philip et al. (1954) demonstrated a quantitative relationship between dosage and incubation period. They used a twenty percent suspension of lymph nodes of an infected dog in sterile milk. This suspension was titrated in two tenths percent bovine albumin in buffered saline. Five ml injections of respective ten-fold dilutions of ($10^{-2.7}$ to $10^{-6.7}$) were injected into five beagle littermates at 60 days of age.

All were fatally infected, but showed increasing incubation periods apparently correlated with increasing dilution. Philip (1955) reported that Neorickettsia helminthoeca in 20 percent node suspension in sterile milk could be lyophilized. After three and one-half months, injection of reconstituted suspension resulted in the death of three dogs. Sporadic, inconsistent transmission by ticks including one instance of transovarial passage to a succeeding generation was found to occur.

A mechanism to explain the intense rate of infection in fluke-bearings fish was proposed by Philip (1958a). He noted that transovarial transmission in the eggs of the fluke and the polyembryonic proliferation of the fluke itself with resultant multiplication of the disease agent could explain the high infection rate. Rickettsialike organisms were located in the shell gland tissues of adult flukes sectioned in situ. The flukes did not appear to be affected by these organisms.

According to Philip (1958b) neither human cases of salmon poisoning disease nor fluke parasitism in man had been reported in the endemic area. He ate portions of raw, infected Oregon trout, aliquots of which caused a fatal infection when fed to a dog, with no ill effects, though a few fluke eggs were passed ten days after ingesting the fish.

Philip (1959) established that infectious node suspensions caused infection when rubbed on abraded skin, but not when instilled in the eyes or fed massively to susceptible dogs.

Farrell (1964, 1966) discovered a second disease, Elokomin fluke fever, which was found to be transmitted also by Nanophyetus salmincola. Serological studies using complement fixation and neutralization tests have established that the disease is distinct from salmon poisoning with no cross protection occurring. Farrell (1968) stated that the condition referred to as salmon poisoning disease is a complex caused by Neorickettsia helminthoeca and the Elokomin fluke fever agent, a rickettsia-like organism. The elementary bodies of Elokomin fluke fever, as seen in lymph node smears, resemble those of Neorickettsia helminthoeca. The incubation period was found to be nine to twelve days. The febrile reaction differs from salmon poisoning disease in that a plateau-type fever occurs instead of the sharply peaked curve seen in salmon poisoning.

Nyberg, Knapp and Millemann (1967) were able to produce salmon poisoning in canines by intraperitoneal injection of washed, ground fluke eggs. This finding demonstrated that salmon poisoning was transovarially transmitted. Nanophyetus salmincola, therefore, serves both as a vector and a reservoir of the disease

Salmon poisoning disease was believed to be non-communicable and historically, precautions were not taken against kennel exposure.

Bosman, Farrell and Gorham (1970) demonstrated that non-endoparasitic transmission of salmon poisoning disease resulted when dogs were exposed by aerosolization or rectal administration to lymph node suspensions or rectal mucosa homogenates.

The intracytoplasmic location of Neorickettsia helminthoeca and its inability to grow on artificial media were prominent factors preventing further research into the etiology of salmon poisoning disease. Little information was available on the interaction of this intracellular pathogen and the in vivo host cells which supported its subsequent multiplication. Morphological studies of Neorickettsia helminthoeca have been limited to light microscopic studies of fixed and stained materials. An in vitro system was necessary which could be useful in the study of the morphology, multiplication and growth of this rickettsia.

The present study includes:

1) the in vitro cultivation of Neorickettsia helminthoeca
2) attempted application of quantitative methods to the study of the multiplication of the rickettsiae
3) the relationship of the Elokomin fluke fever agent to salmon poisoning disease
4) an ultrastructural analysis of Neorickettsia helminthoeca
5) the fulfillment of Koch's postulates as completely as possible for an organism which is an obligate intracellular parasite.

Phase cinemicroscopy of infected tissue culture cells supported by fluorescent and electron microscopy provided a system for observations of living rickettsiae.

METHODS AND MATERIALS

Metacercarial Cysts

Metacercarial cysts of Nanophyetus salmincola were isolated from kidney tissue of Salmo gairdneri from the Alsea River Trout Hatchery of the Oregon State Game Commission. The fresh kidneys were placed in a blender with 200 ml of dechlorinated water and blended for 30 seconds. The homogenate was washed through a sieve which had a standard screen scale of 65 meshes to the inch into a larger finger bowl and allowed to settle for one minute. The supernatant was decanted; the settled material diluted to 1,000 ml with water and allowed to settle in a pharmaceutical flask for five minutes (Nyberg, 1967). The resulting sediment was examined for cysts. The number of cysts was determined from an average of five 0.1 ml aliquot samples of a 50 ml homogenate. Experimental infections of dogs were the result of the administration of 1,000 metacercariae with food.

Rickettsial Strains

Neorickettsia helminthoeca isolated from dogs experimentally infected with metacercarial cysts of Nanophyetus salmincola was used in the major portion of this work. In addition, spleen tissue infected with the Elokomin fluke fever agent or

Neorickettsia helminthoeca was obtained from the laboratory of Dr. Keith Farrell of the Endoparasite Vector Pioneering Research Laboratory, College of Veterinary Medicine, Washington State University, Pullman, Washington. These agents were used in animal cross protection studies.

Dog D-1 was experimentally infected with 1,000 metacercarial cysts of Nanophyetus salmincola. D-2 served as a non-infected control. Body temperatures and weights were recorded daily.

Dogs D-3 and D-4 were infected intraperitoneally (Figure 1) with a two ml homogenate in Snyder I solution (Elisberg and Bozeman, 1964) recovered from a dog experimentally infected with the Elokomin fluke fever agent. A non-infected control dog, D-5, was injected with a non-infected spleen homogenate. During the early febrile period infected dogs were treated daily with 50 mg per pound of tetracycline hydrochloride per os (Polyotic, American Cyanimid) until afebrile for 48 hours. Forty-two days after antibiotic therapy dog D-4 was challenged with a two ml Elokomin fluke fever spleen homogenate. Dog D-3 was given 1,000 metacercarial cysts of Nanophyetus salmincola. Dog D-6 served as a non-infected control. All dogs used in isolation attempts of Neorickettsia helminthoeca were similarly infected with the Elokomin fluke fever agent, given antibiotic therapy, challenged and then given 1,000 metacercariae of Nanophyetus salmincola.

Figure 1. Schema of experiments used to determine the relationship between Neorickettsia helminthoeca and the Elokomin fluke fever agent, and the procedures used in the isolation cultivation and characterization of Neorickettsia helminthoeca.

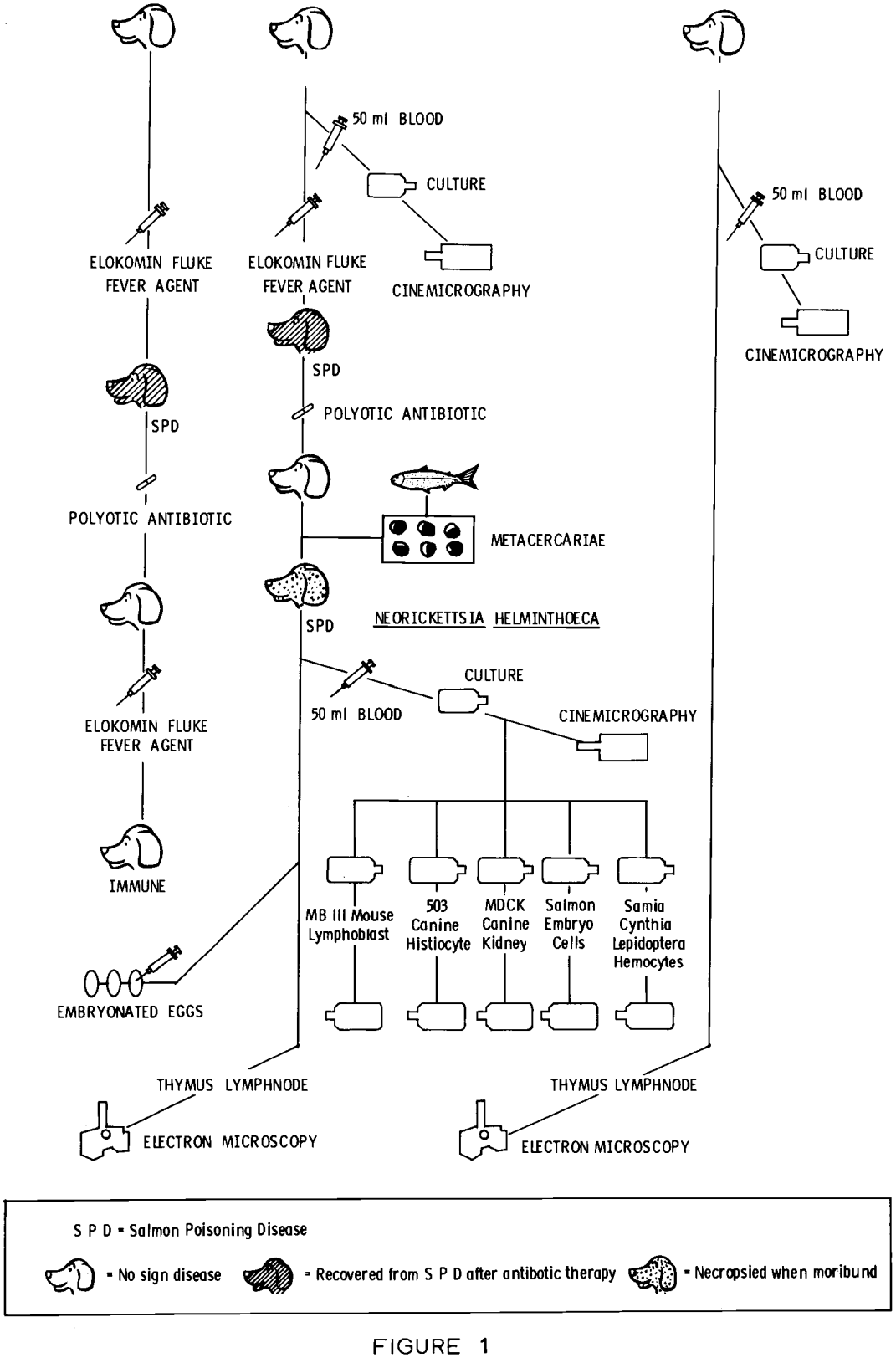

50 ml BLOOD
CULTURE
CINEMICROGRAPHY
ELOKOMIN FLUKE FEVER AGENT
SPD
POLYOTIC ANTIBIOTIC
METACERCARIAE
NEORICKETTSIA HELMINTHOECA
IMMUNE
EMBRYONATED EGGS
MB III Mouse Lymphoblast
503 Canine Histiocyte
MDCK Canine Kidney
Salmon Embryo Cells
Samia Cynthia Lepidoptera Hemocytes
THYMUS LYMPHNODE
ELECTRON MICROSCOPY
S P D = Salmon Poisoning Disease
= No sign disease
= Recovered from S P D after antibotic therapy
= Necropsied when moribund

FIGURE 1

Experimental Canid Hosts

Dogs used in these experiments were obtained from the Benton County pound in Corvallis, Oregon. Kennel procedures similar to those reported by Anderson (1964) were used for the maintenance of the experimental animals. All of the dogs were kept in isolated air conditioned cages designed to minimize cross infections. Body temperatures and weights were recorded daily at approximately 9:00 a.m. All of the dogs used in these experiments were of mixed breeds. The medical histories and vaccination records were not available.

Canine Leucocytes

Canine leucocytes were isolated throughout the experimental period from control, immune and experimentally infected dogs. The leucocytes were isolated and cultured by one of the following methods:

1. Twenty to fifty ml of non-coagulated blood were mixed with 1/4 volume of Alsevers solution and agitated well.
2. The mixture was centrifuged at 2500 rpm for 20 minutes. The WBC layer and an equal layer of the top RBC were removed.
3. Ten ml. of Alsevers solution were added to the blood cells in a conical centrifuge tube and centrifuged at 2500 rpm for 15 minutes. The top layer was removed as before and an attempt was made to leave the RBC layer.

4. Ten ml of Alsevers were added and centrifuged as before.
5. Only the WBC layer was removed to which 2.5 ml of Medium 199 (Grand Island Biological) with 10% fetal bovine serum (Microbiological Associates) was added.
6. One ml of the WBC suspension was inoculated onto the tissue culture cells which had been rinsed three times previously.
7. The cells were incubated at 35°C for 10-14 days and passed blindly.

The isolation procedure was later modified (Nyindo et al., 1971) and consisted of the following:

1. Approximately 50 ml of blood were obtained from either infected, immune or control dogs in a plastic syringe which contained 3000 units of sodium heparin (Riker Laboratories).
2. The syringe was inverted (needle up) for 60 minutes at 22°C. to allow the erythrocytes to sediment. Leighton tubes (Belco) with 50 x 10 mm cover slips and 30 ml tissue culture flasks (Falcon Plastics) were inoculated with two and six aliquots, respectively.
3. The cultures were incubated at 37°C. for 48 hours to allow the WBC to attach to the coverslips and plastic flasks.
4. The fluids were discarded and the cultures rinsed once with normal dog serum (Grand Island Biological) and once with Hank's balanced salt solution.

5. Culture medium consisting of eighty percent Eagle's minimum essential (Microbiological Associates), 0.1 mM L-glutamine per ml and twenty percent canine serum (Grand Island Biological) was added in two and six ml volumes to the Leighton tubes and culture flasks, respectively. The medium was replaced every 48 hours. Culture fluids from infected cultures were used for in vitro and in vivo passage.

Light Microscopy

Smears of whole blood or isolated WBC were prepared and stained with Gimenez or acridine orange stains. Liver, pancreas, spleen, lung, thymus and mesenteric lymph node tissues of experimentally infected and non-infected dogs were removed aseptically, fixed in buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin eosin. Impression slides of mesenteric lymph node and spleen were prepared according to the technique of Mori (1964) from experimentally infected and non-infected control dogs. Impression slides were stained using Machiavello's stain, May-Grunwald's stain, Gimenez (1964) stain and acridine orange fluorescent stain (Nyberg, 1967).

Transmission Electron Microscopy

Mesenteric lymph node and thymus tissue of experimentally infected and non-infected control dogs were used for transmission electron microscopy. Tissues were immediately separated from surrounding adipose and connective tissues and minced. The tissue was fixed in 5% glutaraldehyde in Sorenson's buffer (pH 7.0, osmolarity 0.125 M), stained with one percent osmium tetroxide, dehydrated in a graded acetone series, embedded in epon araldite and sectioned on a Porter-Blum MT-2 ultramicrotome with a diamond knife. Electron micrographs were obtained using a Philips EM 300 transmission electron microscope and were subsequently photographically enlarged.

Time-Lapse Cinemicrography

Cinemicrographic records (Eastman Double-X negative or Tri-X 16 mm) were prepared of control and experimentally infected tissue cultures. Rose chamber preparations maintained at 35°C. or room temperature were photographed at a time lapse interval of 12 seconds. After recording the appearance and activity of control cultures, the chambers were perfused by gravity flow with 20% suspensions of tissues infected with Neorickettsia helminthoeca or the culture fluid harvested from infected monocyte cultures.

Preservation of Tissues in Liquid Nitrogen

Samples of infected mesenteric lymph nodes and splenic tissues were minced into small cubes, sealed in separate sterile ampules and frozen in liquid nitrogen.

Primary Tissue Cultures

Experimentally infected and non-infected control spleen, thymus and mesenteric lymph nodes were used to establish primary cultures as follows:

1. Tissues were removed and transferred aseptically to a sterile petri dish and washed with calcium and magnesium free phosphate buffered saline (CMF-PBS).
2. The tissue was cut into two to four mm pieces and transferred to a sterile trypsinization flask. Sterile trypsin solution 0.25% (Difco) was added. Ths tissues were trypsinized for 20 minutes and centrifuged at 600 rpm for two minutes to separate cells from the erythrocytes contained in the supernatant. This was repeated until the media was free of erythrocytes.
3. When the erythrocytes were removed, the tissue was resuspended in trypsin solution and incubated at 4^{o}C. with constant stirring for six hours. The cells were centrifuged for 15 minutes at 13,000 rpm. The supernatant fluid was discarded and fresh

trypsin added. Trypsinization was continued until the tissues were completely dissociated.

4. The cells were filtered through two layers of sterile cheese cloth and washed three times in CMF-PBS, suspended in CMF-PBS and an aliquot examined for the number of cells present.

Isolation of the Rickettsia from Experimentally Infected Tissue

Fresh or frozen spleen, thymus or lymph node tissues were used to isolate Neorickettsia helminthoeca.

1. A 20% suspension of the infected tissue was prepared in brain heart infusion (Difco), Earl's balanced salt solution with one percent bovine serum albumin (Difco) or Snyder's I solution with one percent bovine serum albumin.
2. The suspension was centrifuged at 2000 to 3000 rpm for 30 minutes. The supernatant was used to inoculate mice, rats, chicken yolk sacs and tissue cultures.

Isolation Procedures for Rickettsial Agents in Mice, Rats and Guinea Pigs

Mice, 12-15 grams, were each inoculated with one ml intraperitoneally and held for 21 days. If the mice appeared moribund at six to ten days post infection, one was necropsied. If they all appeared normal, they were autopsied at 21 days. Mice were killed with ether

and disinfected with alcohol. The abdominal cavity was cut open and an impression slide was prepared of the spleen. A 20% suspension of the spleen was prepared and passed into another mouse.

Young rats were also used in later experiments following the same protocol.

Immunosuppressants

A 20% infected organ suspension was injected into guinea pigs which had been treated intraperitoneally one, two, three, or four days previously with three mg of cortisone acetate (Sigma). However, one group received the cortisone and suspension simultaneously. All guinea pigs were given three mg cortisone twice in the following week.

Isolation Procedure for Rickettsiae in Yolk Sacs

Chicken eggs six days old were disinfected with iodine. The yolk sac was inoculated with a 20 percent lymph node suspension or infected culture fluid.

1. A small hole was punched over the air space, off center away from the embryo.
2. Under sterile conditions a one ml syringe fitted with a 22-gauge needle, one inch long, was used to inoculate 0.2 ml of the organ suspension or culture fluid into the yolk sac.

3. The area of inoculation was wiped with disinfectant and sealed with Duco cement.

4. Control eggs were inoculated with rickettsia-free organ suspension or non-infected culture fluids.

5. Eggs were labeled with name of material, specimen number and date.

6. The eggs were incubated at 35°C. and candled daily. Embryos dying 48 hours after inoculation were discarded and not considered. All yolk sacs from embryos dying after three days were saved and harvested.

7. When the eggs were 16 to 19 days old the yolk sacs were harvested for blind passage.

The yolk sacs were harvested by chilling the eggs in the refrigerator for one hour.

1. Iodine was applied to the shell and wiped away with alcohol.

2. The shell was cracked to expose the embryo and yolk sac.

3. Several yolk sac areas approximately 1.5-2.0 cm square were placed in a sterile vial.

4. A sample of yolk sac was placed on a paper towel and a one mm square section removed.

5. The one mm square was then placed between two slides and a moderate amount of pressure was applied to separate the yolk sac into individual cells.

6. The yolk sac cells were streaked over the slides by sliding one slide over the other.

7. The slides were dried 24 hours before staining.

Mycoplasma Isolation

Attempts were made to isolate any possible mycoplasma present in organ suspensions and tissue culture fluids containing Neorickettsia helminthoeca. The media used were obtained from the laboratory of Dr. James O. Stevens of the Department of Veterinary Medicine, Oregon State University, Corvallis, Oregon. The three culture media were used to isolate M. synoviae, M. gallisepticum and M. melignides, respectively. The composition of the media was not known as this information was not published. Cultures were inoculated with 0.3 ml of the specimen to be tested and incubated at 37°C. and observed daily.

Tissue Culture Methods

Primary cultures were obtained from canine leucocytes, lymph nodes and thymus. Chicken embryos and salmonid eggs were utilized to obtain primary cultures of dispersed cells. Established cell lines of CCL34MDCK canine kidney, 503 canine histiocyte, CCL32MBIII mouse lymphoblasts and Samia cynthia (lepidoptera) hemocytes were kept in continuous culture throughout this study. Photographic records were kept of control and infected cultures.

Chicken Fibroblasts

Chicken fibroblasts were obtained from eight-ten day embryos following the procedure for chick embryo cell culture of Schmidt (1969).

Salmonid Cells

Salmon cells were obtained from the embryos of the steelhead trout, Salmo gairdneri. Eggs were collected from the Alsea Fish Hatchery, Oregon State Fish Commission, Alsea, Oregon. The embryos were dissociated in 0.25% trypsin (Fryer et al., 1965) in Hank's BSS at a pH of 7.4. Cells were dissociated by treating them for 15 minutes at 15°C. with the trypsin solution. The resulting fluid and cells were discarded and fresh trypsin added. Cells were then removed at 30 minute intervals until dispersion was complete. They were then removed from the trypsin solution by centrifugation at 600 rpm for 20 minutes and suspended in the growth medium:

Eagle's minimum essential medium	80%
Agamma calf serum	20%

Cells were then counted and inoculated into 30 ml tissue culture flasks (Falcon Plastics) and kept undisturbed at 20°C. for 48 hours. They were subcultured by the enzyme dispersion method and were dispersed by removing them from the flasks and treating them with

0.25% trypsin followed by pooling, washing, and resuspension in culture medium. The culture fluid was changed every 12 days.

Canine Kidney Cells

The MDCK cell line was derived from a kidney of an apparently normal adult male cocker spaniel, September, 1958, by S. H. Madin and N. B. Darby. The cells were submitted to the American type culture collection (Rockville, Maryland) in the 49th passage. One frozen ampule of cells from passage 54 was obtained from the American Type Culture Collection on September 16, 1970. The cells were promptly thawed and have been kept in continuous culture with a subculture interval of six days. Stock cultures were maintained in 30 ml tissue culture flasks (Falcon Plastics). Growth has occurred in a mixture of 0.5% lactalbumin hydrolysate (Nutritional Biochemicals) in Earle's BSS with reduced bicarbonate (0.85 gm/l), 95%; fetal bovine serum, 5%; without antibiotics. Culture medium was renewed twice weekly. The cells were subcultured before reaching confluency (about six to seven days). Subculturing procedure consisted of rinsing the cell sheet twice with ATV solution (NaCl, 8 gm/l; KCL, 0.4 gm; dextrose, 1 gm; $NaHCO_3$, 0.58 gm; trypsin Difco 1.250, 0.5 gm; versene disodium salt 0.2 gm; distilled water to 1,000 ml), removing the ATV and allowing the culture to stand at room temperature for 20 minutes. Fresh culture medium was added and the cells dispensed

into new flasks. The subcultivation ratio was 1:2 to 1:4.

Canine Sarcoma Cells

The 503 sarcoma cell line was derived by Albert Samso from the Sticker sarcoma, a transplantable canine venereal tumor, originally carried as a subcutaneous transplant in dogs. The histiocyte-like cells have been cultured continuously in medium 199 with Earl's balanced salt solution with 20 percent fetal bovine serum. Passages were made by gentle trituration of confluent monolayers after trypsinization. The stock culture was obtained from the laboratory of Dr. H. James Akiyama, Department of Biological Sciences, Microbiology Area, San Jose State College, San Jose, California.

Mouse Lymphoblasts

The MB III strain of cells was derived in 1947 by W. M. deBruyn and G. O. Guy as one of three cells strains (MB I, II and III) with tumor-producing and non-tumor-producing properties initiated from a mouse lymphosarcoma MB (T86157) which originated in 1935 as a spontaneous tumor that arose in an F_1 hybrid of the dilute brown Murray-Little strain and C57 black strain. The cells lost their malignancy for tumor-susceptible mice and grow either as a monodispersed suspension of individual cells in the fluid medium or as monolayers. Stock cultures were maintained at 37°C. in a volume of

1.5 ml in 15 x 125 mm Kimax screw cap test tubes and rotated in a roller drum (Wyble Engineering Developments Corp.) at 12 revolutions per hour. Passages were made by gently shaking the culture for a moment to free attached cells, removing half of the cell-containing fluid and then adding an equal volume of fresh nutrient. The cell suspension removed from the culture tube was discarded or used to establish a duplicate passage line. Cells were also cultued in 30 ml tissue culture flasks (Falcon Plastics) in three ml of medium.

The nutrient medium consisted of the following:

Eagle's MEM, with non-essential amino acids, sodium pyruvate and Earl's BSS, 90% (Microbiological Associates), Fetal bovine serum screened for cytopathic bovine viruses and non-cytopathic BDV 10% (Microbiological Associates), Five to ten percent CO_2 in air to maintain the pH at approximately 7.2-7.4.

Tissue culture medium was renewed two-three times weekly with a subculture interval of six to seven days. The subculture procedure involved shaking or gentle mopping of cells from the floor of the culture vessel. A subculture ratio of 1:2 to 1:4 was used.

Lepidopteran Hemocytes

The lepidopteran hemocyte cell line was established from hemocytes of the pupa of Samia cynthia in September of 1968 by Dr. Jowett

Chao, Department of Zoology, University of California at Los Angeles. The cells were grown both as free and attached cells.

The nutrient medium consisted of the following:

Grace's Insect T. C. Medium without insect plasma 90% (Grand Island Biological)

Fetal calf serum, heat inactivated 10% (Grand Island Biological).

Culture medium was renewed at 10-14 days with a subculture interval of the same time. The subculture procedure involved shaking or gentle trituration of cells from the vessel or simply changing the medium.

Preparation and Infection of Cells

Stocks of cells were increased, pooled and enumerated prior to use. A quantity of the pool containing sufficient cells for the experiment was centrifuged at 1000 rpm for five minutes. The sedimented cells were gently resuspended and mixed with a rickettsial suspension or inoculated with fluids from infected cultures. The infected cell suspension was diluted with culture medium to provide the final concentration. Non-infected control cultures were treated in a similar manner except culture medium was added in place of the rickettsial suspension. Infected cultures were examined daily for 14 days utilizing the following techniques: (1) enumeration of cell population,

(2) microscopic observation of quantities of intracellular rickettsiae in stained smears of cultured cells. Fluids from infected cultures (2-8 ml volumes) were inoculated intraveneously into susceptible dogs. Blood from inoculated dogs was used to reisolate the organism in cell cultures.

Fate of Inactivated Rickettsiae

Fluids from infected cultures were harvested and pooled in a sterile screw cap test tube. The resulting rickettsial suspension was held at 56°C. for 60 minutes. Primary cultures of dog leucocytes or sarcoma cells 503 were then inoculated with the heat treated cells.

RESULTS

Observations of Experimental Infections

Experimental infection of dogs with metacercarial cysts of Nanophyetus salmincola resulted in the development of signs of salmon poisoning disease. The incubation period was five to seven days post ingestion of cysts. The onset of signs was rapid (Figure 2) with a sharp rise in temperature, loss of appetite and general malaise. Body temperatures (Figure 2) then fell rapidly to sub-normal until death occurred. The course of the disease was from seven to ten days. Food was not ingested after the onset of the signs with a resultant loss of weight. Diarrhea commenced at the time of peak febrile response and resulted in severe dehydration. Numerous eggs of Nanophyetus salmincola were observed five days post ingestion of metacercarial cysts. Non-infected control dogs did not show signs of salmon poisoning disease.

Dog D-1, infected with 1,000 metacercarial cysts of Nanophyetus salmincola, and dog D-2, a non-infected control, were necropsied on experimental day 15. Liver, pancreas, spleen, lung, thymus, and mesenteric lymph node samples were prepared for histopathology. Rickettsiae were localized in leucocytes, primarily macrophages of the spleen, thymus and lymph node tissues. Rickettsiae were not observed in the pancreas, liver or lung tissues. Small

Figure 2. Comparative body temperatures and weights of principal (1,000 metacercarial cysts) and non-infected control dogs.

Figure 3. Comparative body temperatures of principals (infected with the Elokomin fluke fever agent) and non-infected control dogs. Antibiotic therapy given at arrow. E.F.F.A. = Elokomin fluke fever agent.

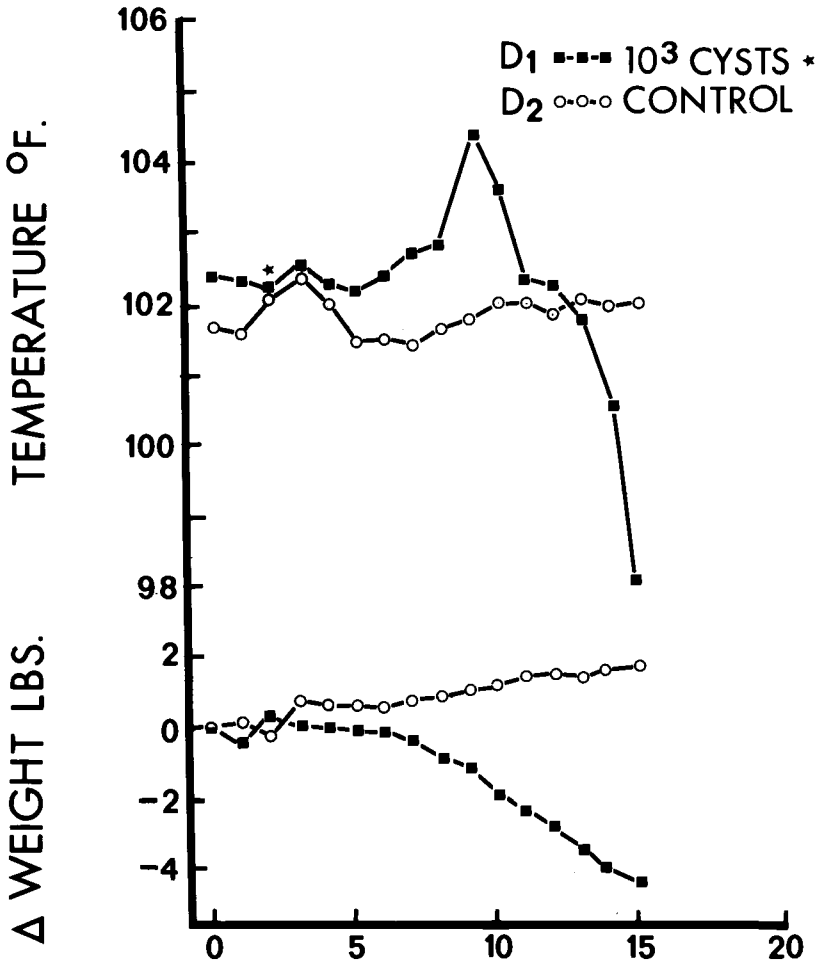


FIGURE 2

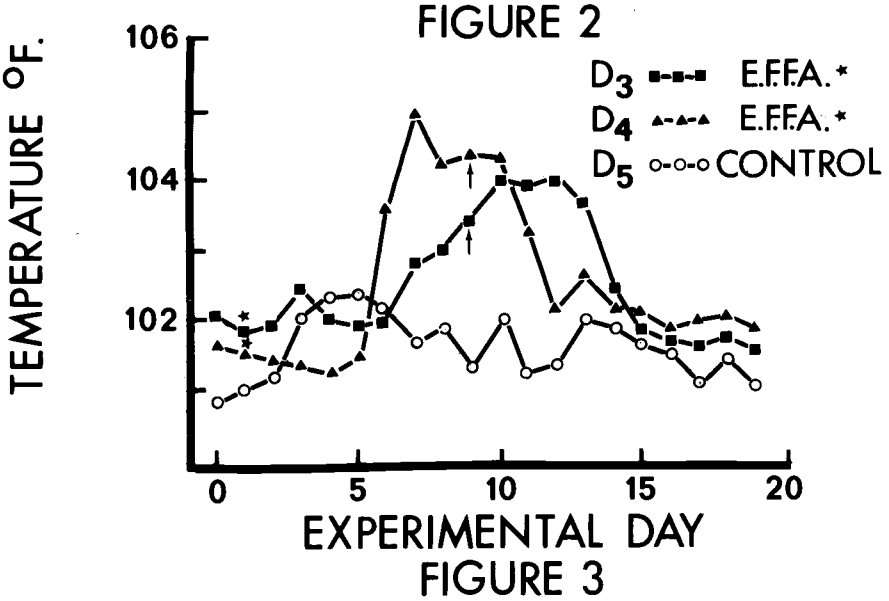


FIGURE 3

hemorrhagic areas were observed in lung tissue. Hyperplasia was found throughout the reticuloendothelial system of infected animals. Rickettsiae and hyperplasia were absent from non-infected control tissues. Many rickettsiae were distributed throughout the cytoplasm of infected lymph node and spleen impression slides.

Rickettsial bodies were stained a bright red against a blue-green cytoplasm when stained with Gimenez stain. Machiavello's staining technique gave similar results with the rickettsiae appearing bright red and the background blue. May-Grumwald and Giemsa stains both resulted in purplish-blue rickettsiae. Acridine orange fluorescent staining indicated the presence of RNA in the cytoplasm of lymphocytes from infected dogs. RNA was demonstrated in circulating lymphocytes in conjunction with the onset of the febrile period. Control lymphocytes did not stain orange indicating detectable amounts of RNA were absent. The nuclei of lymphocytes from infected and non-infected animals fluoresced green indicating DNA.

Dogs D-3 and D-4 which were infected intraperitoneally with 2 ml of a spleen homogenate recovered from a dog experimentally infected with Elokomin fluke fever both developed signs of Elokomin fluke fever. Four days after inoculation a plateau-type febrile response resulted (Figure 3) and anorexia, diarrhea, dehydration and general malaise were exhibited. Dog D-5, a non-infected control, did not show signs of Elokomin fluke fever. Eight days post

inoculation antibiotic therapy was initiated with the administration of 50 mg per pound of tetracycline hydrochloride to dogs D-3 and D-4. Temperatures returned to normal (Figure 3) and recovery was complete. Forty-two days later dog D-3 was given 1,000 metacercarial cysts of Nanophyetus salmincola and dog D-4 was challenged with an intraperitoneal injection of a 2 ml homogenate of an Elokomin fluke fever spleen. Dog D-3 developed signs of salmon poisoning disease with a sharp rise in temperature followed by decline to sub-normal (Figure 4). Dog D-4 did not demonstrate signs of disease nor did dog D-6, a non-infected control. Dogs D-3, D-4, and D-6 were necropsied on experimental day 70.

Transmission Electron Microscopy

Examination of sections of mesenteric lymph node and thymus from dogs experimentally infected with Neorickettsia helminthoeca revealed the presence of rickettsiae. All of the organisms found were within cells and were limited to the cytoplasm. A phagocytic vacuole, clearly limited by a cell membrane, enclosed groups of rickettsiae (Figures 7, 8, and 9) located in mesenteric lymph nodes. The membrane was not as apparent in thymus tissue. Rickettsial cells were bounded by a cell wall, which surrounded the entire organism, and by the underlying cytoplasmic membrane, which limited the cytoplasm of the organism (Figures 7, 8, 11, and 12). Each of these membranes

Figure 4. Comparative body temperatures of principals (Dog D-3 was given 1,000 metacercarial cysts after recovery from Elokomin fluke fever; Dog D-4 challenged with the Elokomin fluke fever agent after recovery from Elokomin fluke fever) and non-infected control dogs.

Figure 5. Comparative body temperatures of principal (Dog D-7 infected with Neorickettsia helminthoeca from infected tissue culture) and non-infected control dogs.

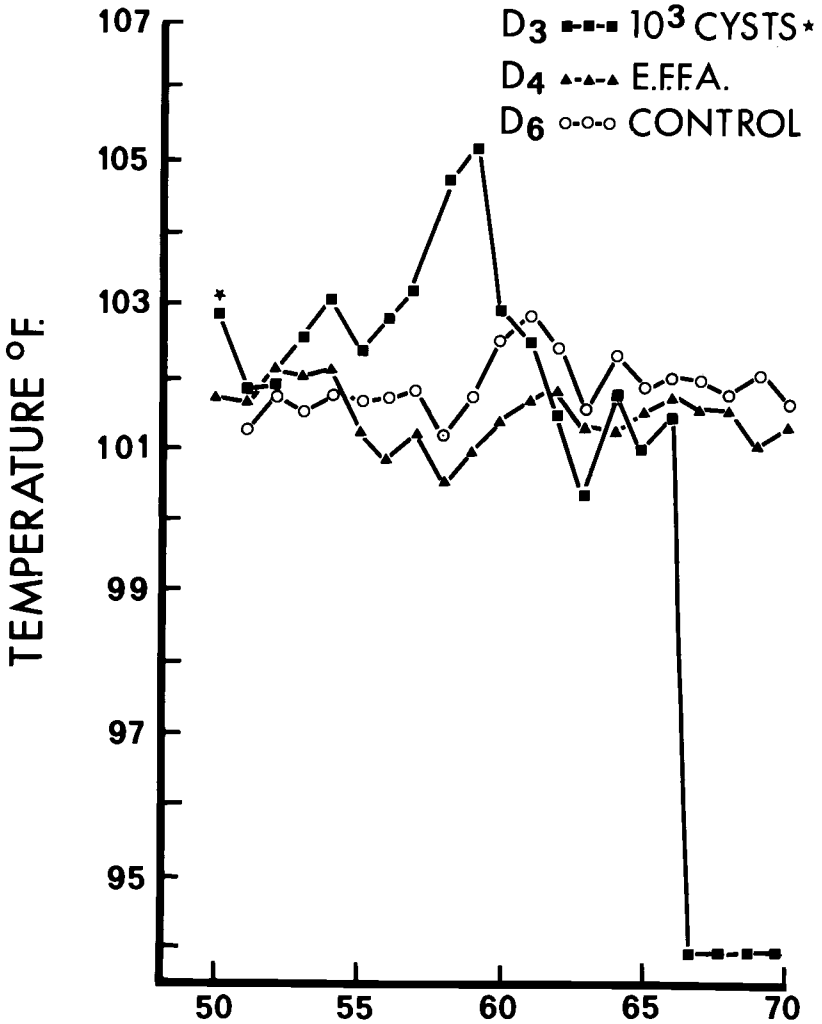


FIGURE 4

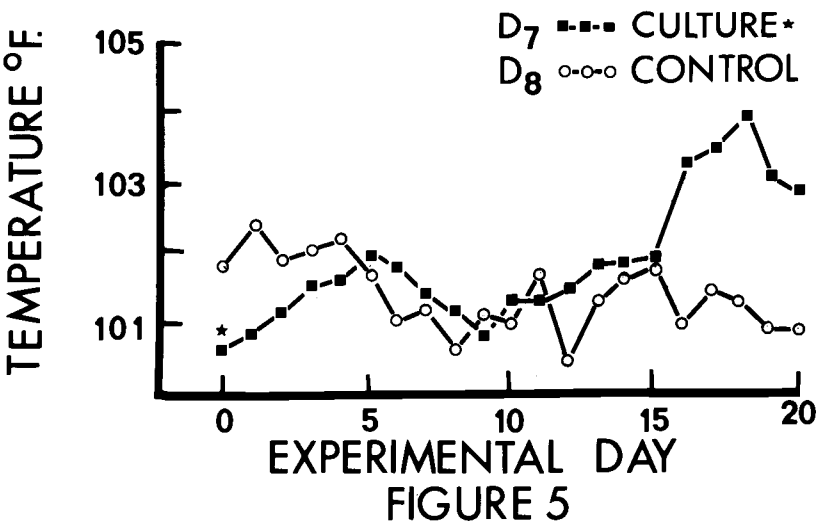


FIGURE 5

Figure 6. Mesenteric lymph node of a non-infected control dog. Mitochondria (m); Nucleus (n). (16,000 x)

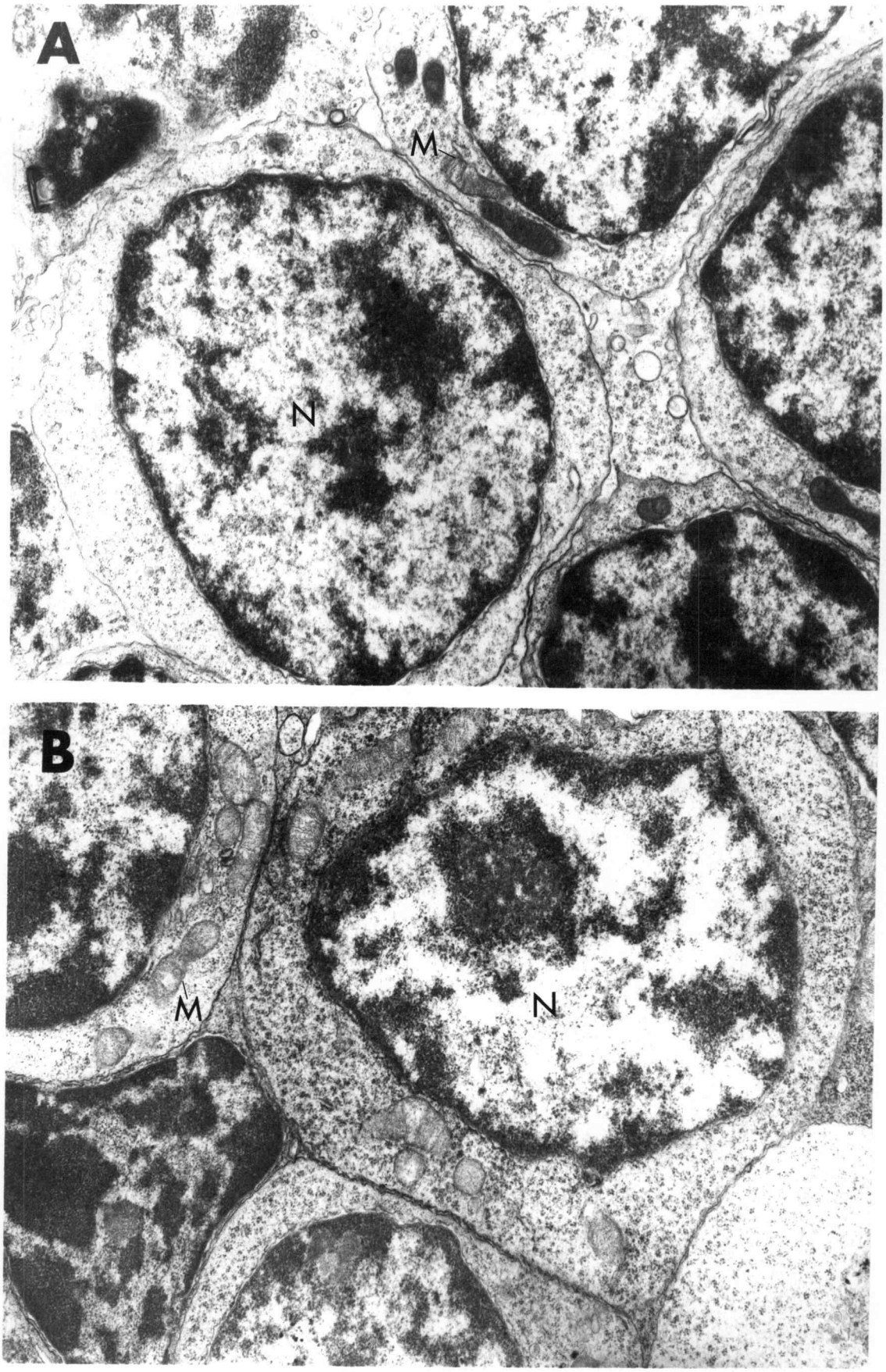
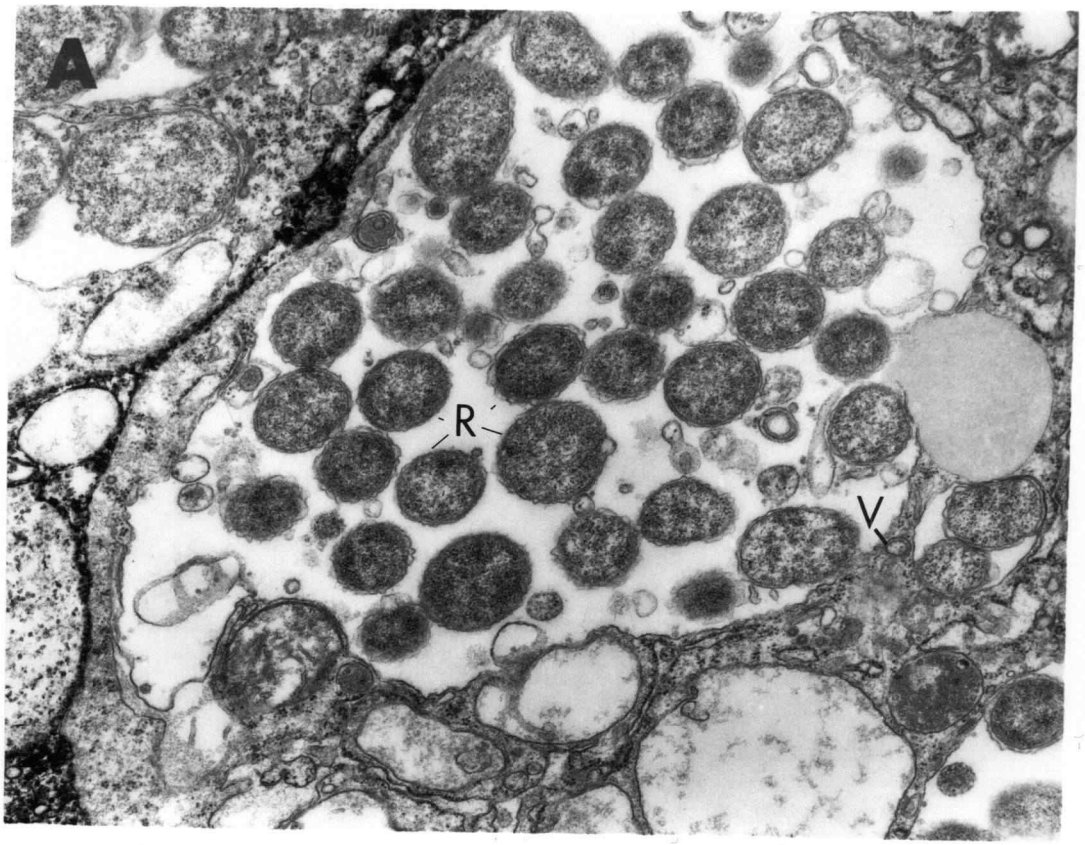

A
M
N
B
M
N

Figure 7. Mesenteric lymph node of a dog infected with Neorickettsia helminthoeca.

(A) Rickettsiae (R); Phagocytic vacuole (v). (20,000 x)

(B) Rickettsiae (R); Phagocytic vacuole (v). (34,500 x)


A
R
V

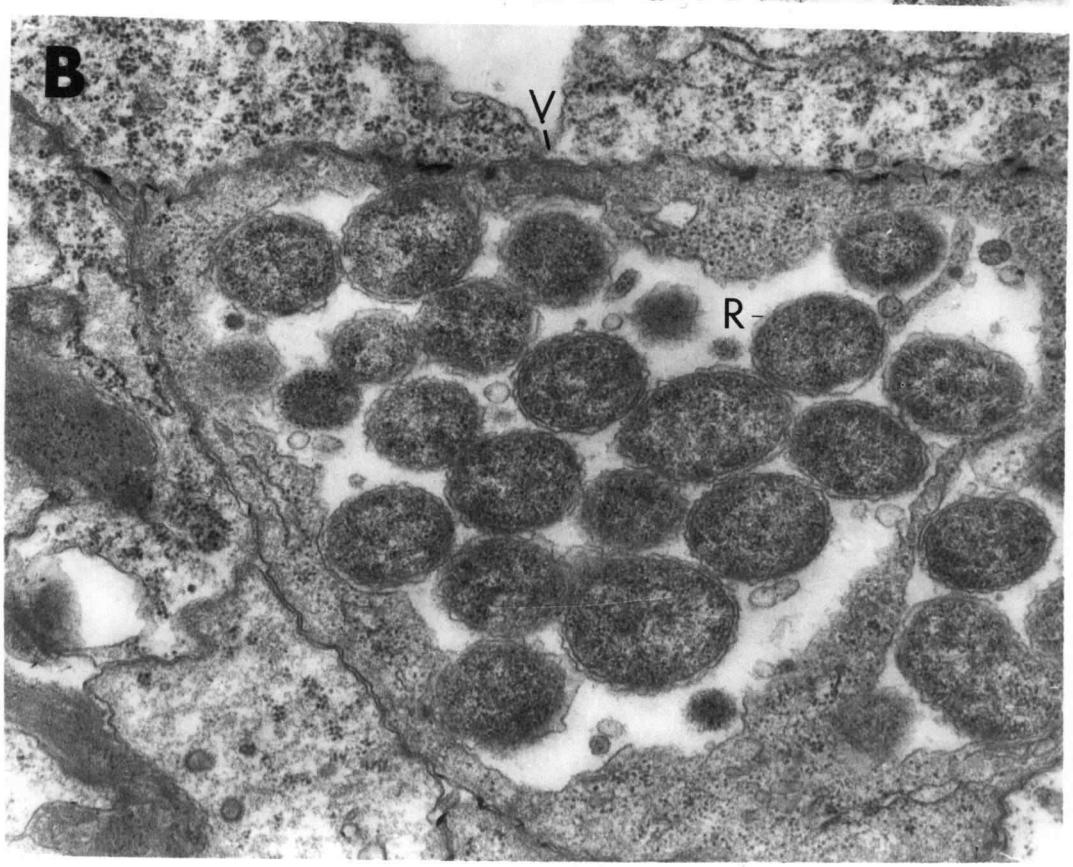

B
V
R

Figure 8. Mesenteric lymph node of a dog infected with Neorickettsia helminthoeca.

(A) During binary fission, a furrow (f) is formed by the constriction of both the cell wall (w) and the plasma membrane (pm). (16,000 x)

(B) Detail from A. (208,000 x)

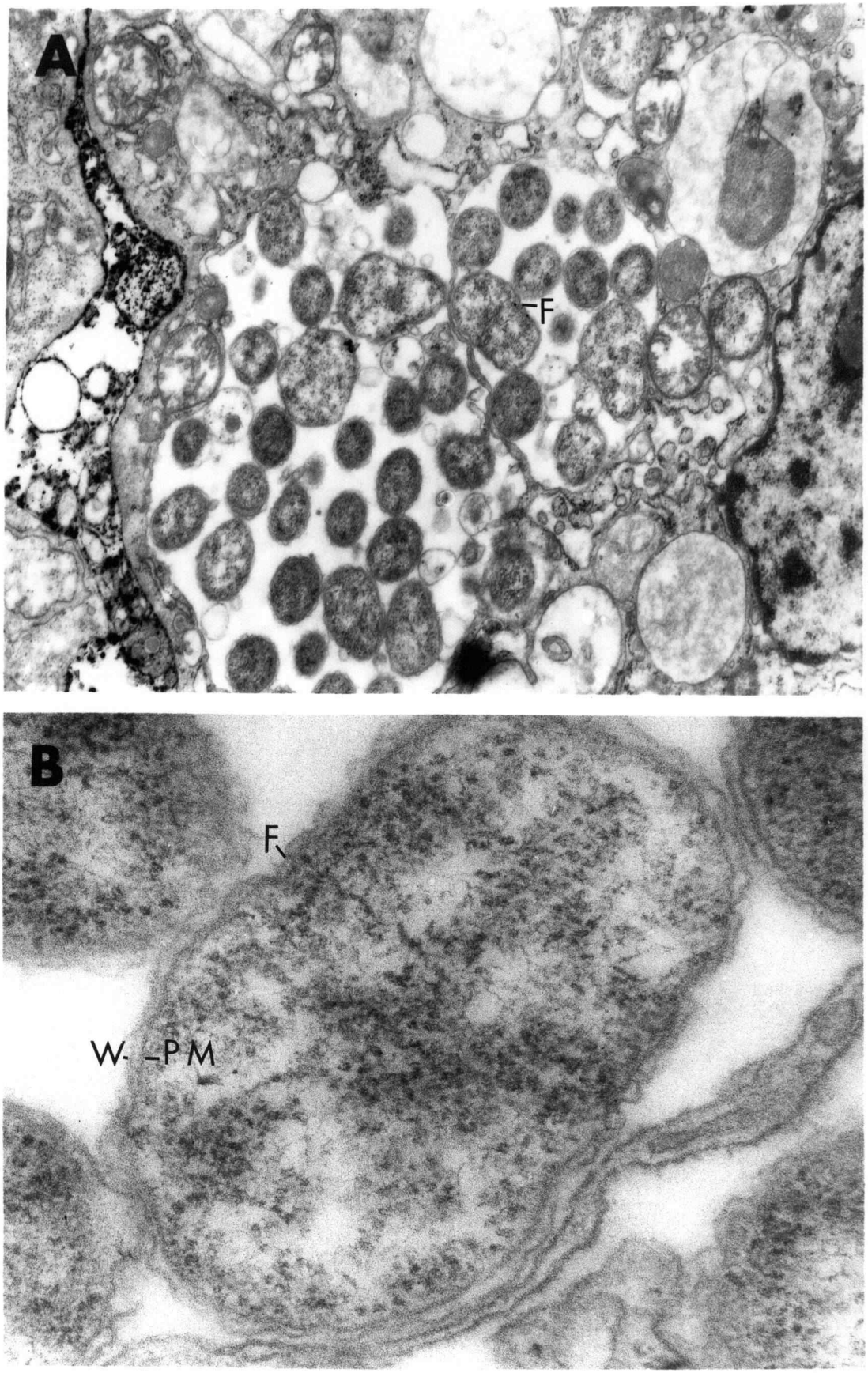

A
F
B
F
W
PM

Figure 9. Mesenteric lymph node of a dog infected with Neorickettsia helminthoeca. A serial section of Figure 8A indicating further development of the division furrow. (28,500 x)

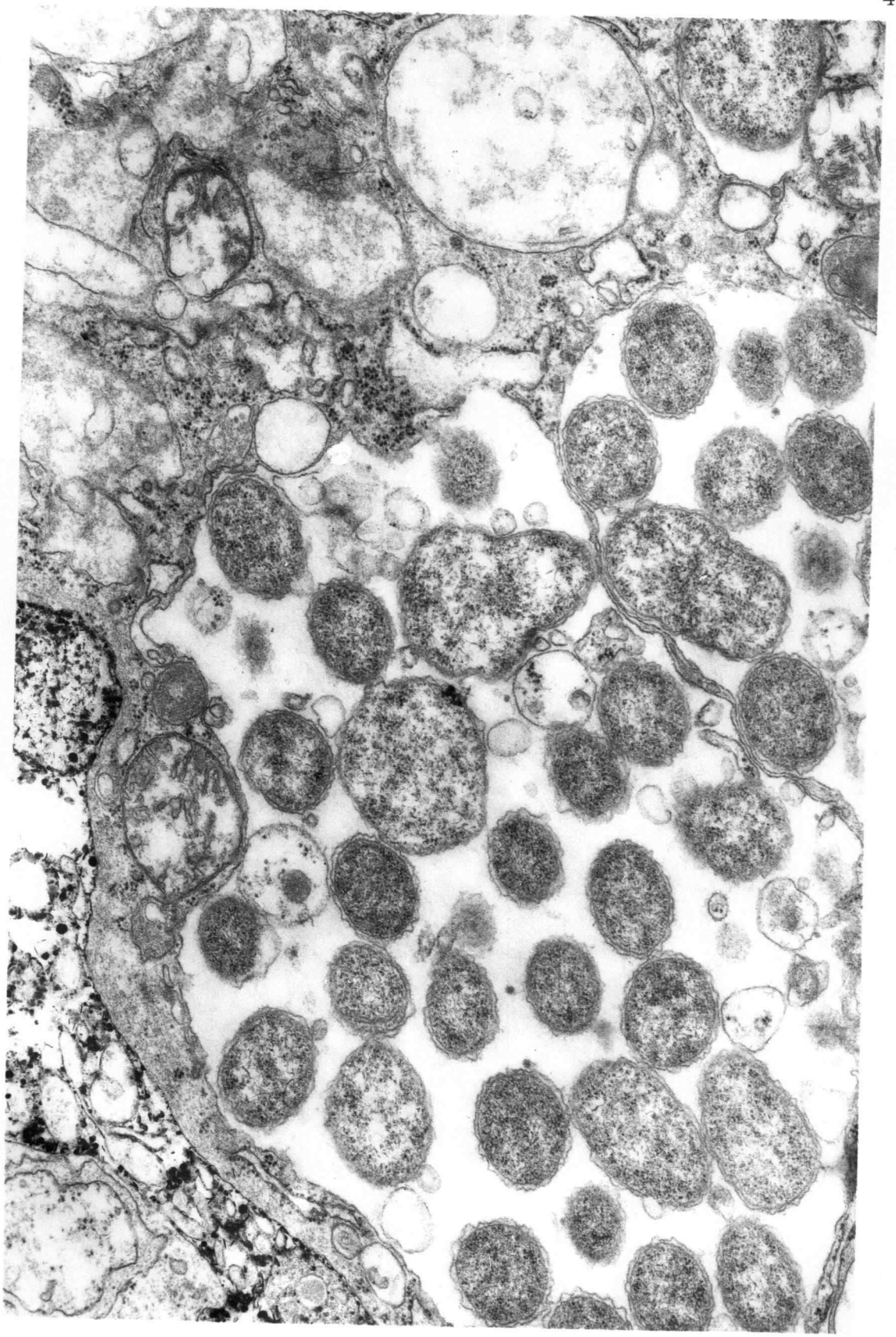
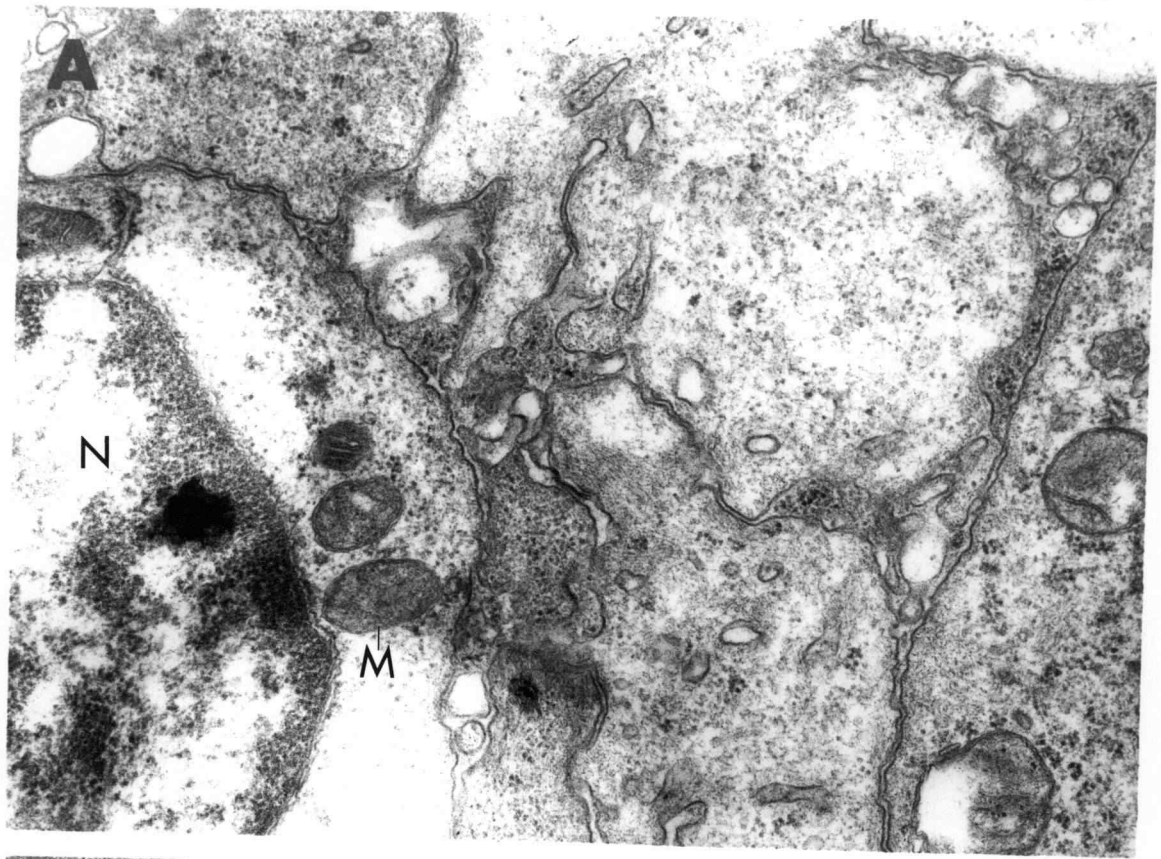


Figure 10. Thymus of a non-infected control dog.

(A) Nucleus (n); Mitochondria (m). (31,000 x)

(B) Nucleus (n); Mitochondria (m). (20,000 x)


A
N
M

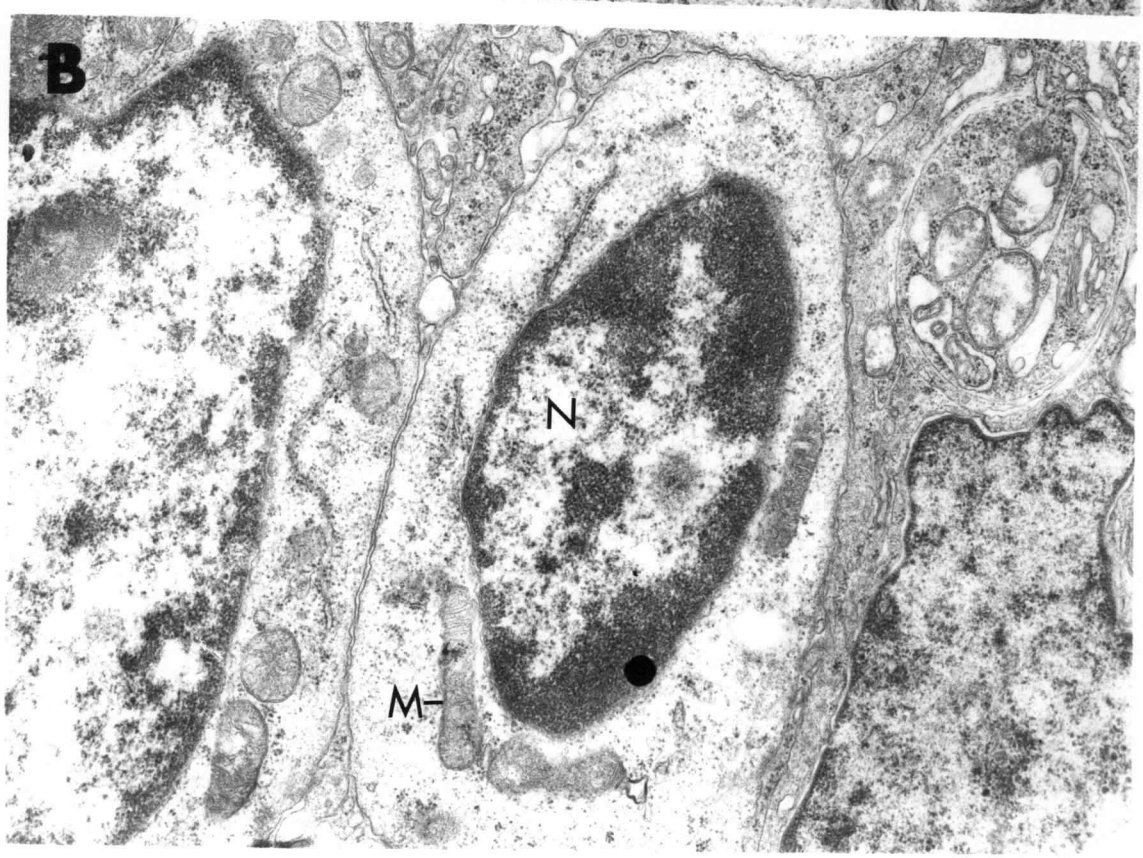

B
N
M

Figure 11. Thymus of a dog infected with Neorickettsia helminthoeca.

(A) Mitochondria (m); Nucleus (n). (11,000 x)

(B) Nucleus (n). (21,000 x)

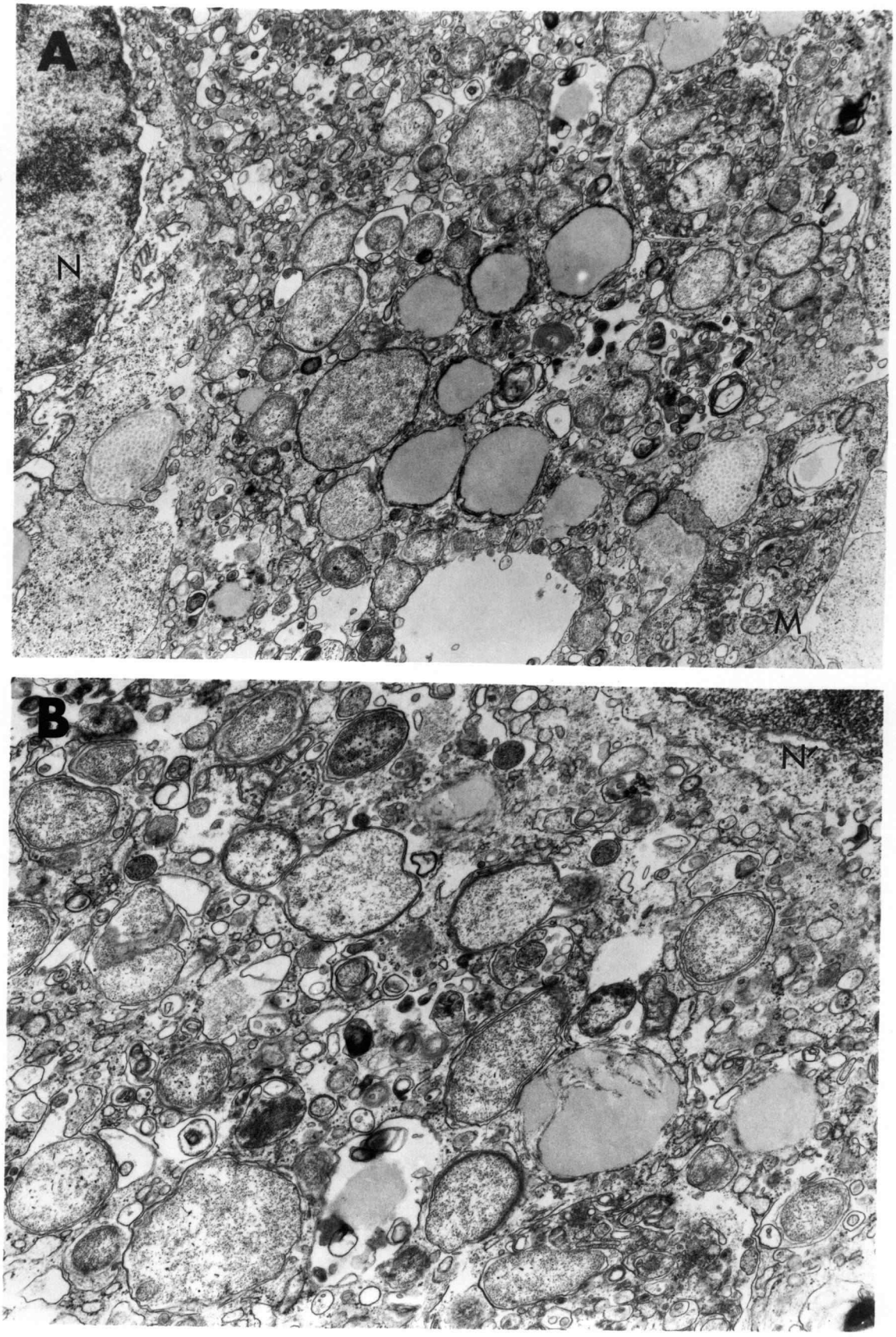
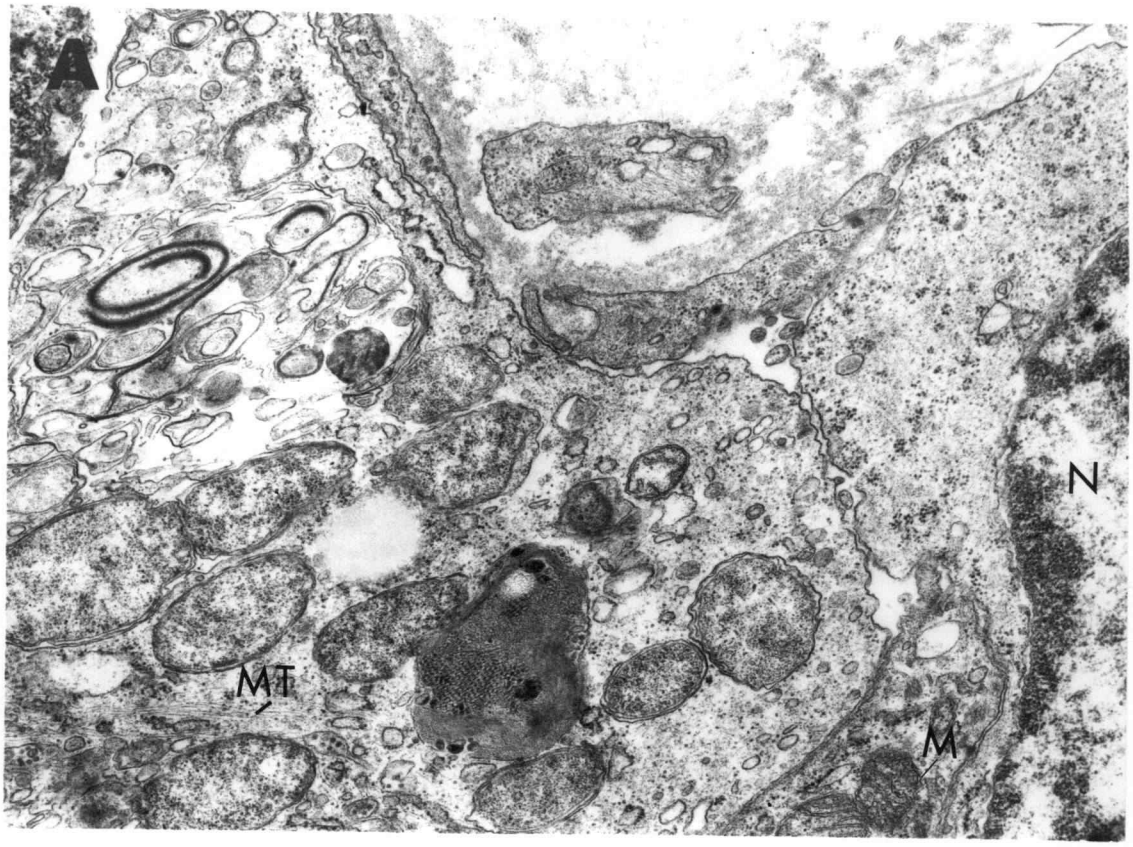
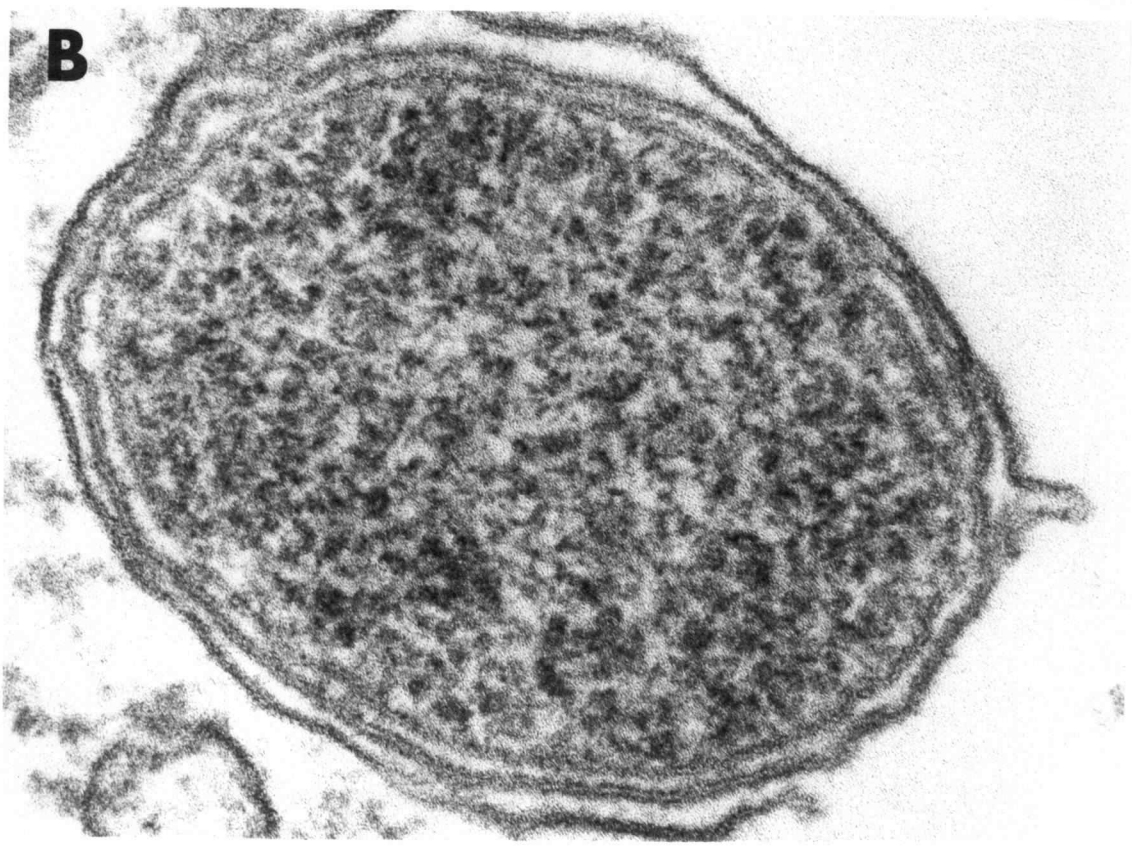

A
N
M
B
N

Figure 12. Thymus of a dog infected with Neorickettsia helminthoeca.

(A) Nucleus (n); Mitochondria (m); Microtubules (mt). (21,000 x)

(B) Higher magnification of a rickettsia. (195,000 x)


A
N
MT
M


B

was a tri-layered structure (two electron dense layers separated by a pale area) and showed the unit membrane structure characteristic of biological membranes (Figures 8B and 12B). The outer cell walls were clearly defined, but the cytoplasmic membranes were sometimes fused with the underlying cytoplasm and, thus, less clearly resolved (Figures 7, 8, 9, 11, and 12). Both the plasma membrane and the cell wall were rippled, which may represent shrinkage during the preparation of the samples. The inner structure of the rickettsial cell was found to have electron dense areas intermingled with pale areas. Structural components encountered inside the rickettsial cells were ribosome-like granules and fine DNA-like strands. The ribosome-like granules were localized in the dense areas, and DNA strands observed in irregular pale areas (Figures 8B and 12B). Individual rickettsia were regularly encountered which were round although most appeared slightly oblong. The mean size of Neorickettsia was 0.5 by 0.7 microns. The plasma membrane and cell wall of some of the larger organisms contained transverse constriction furrows which are characteristic of rickettsiae undergoing binary fission (Figures 8A and 8B). Rickettsiae were not observed in non-infected control tissues (Figures 6 and 10).

Time Lapse Cinemicrography

Monocytes obtained from the jugular vein of non-infected control dogs were photographed in Rose chambers. The cells were active and

did not divide during the period of observation. Selected chambers were perfused by gravity flow with homogenates of tissues infected with Neorickettsia helminthoeca or culture fluid harvested from infected monocyte cultures. Chambers which were perfused with tissue homogenates were not satisfactory because cellular debris and contamination prevented observation. Culture fluid harvested from infected monocyte cultures as an inoculum allowed observation of infected cells. Rickettsiae were recognized in infected cells as bacillary structures. These structures increased in numbers as the infection progressed and were later observed in fixed and stained cells. Rickettsia could not be recognized until 60-70 hours after infection. The organisms were always observed in the cytoplasm. Rickettsia were seen to emerge from infected cells by way of long filamentous microfibrillar structures protruding from the cell surface. Organisms were sometimes released into the culture fluid or the structure was retracted.

Cinemicrographic observations of Neorickettsia helminthoeca are complicated by the low optic contrast in phase contrast microscopy.

Preservation of Tissues in Liquid Nitrogen

Samples of infected lymph node and splenic tissues maintained their infectivity after storage in liquid nitrogen. Frozen ampules of

infected 503 sarcoma cells maintained their infectivity when frozen in cryoprotective medium (basal Medium Eagle with 15 percent dimethyl sulfoxide used 1:1 with suspension medium).

Primary tissue cultures of spleen, thymus and mesenteric lymph nodes from experimentally infected and non-infected control dogs were obtained. Rickettsiae were found within all cell types harvested from infected dogs. Non-infected control tissues did not harbor rickettsiae.

Isolation from Experimentally Infected Tissue

Attempts to isolate rickettsiae from fresh or frozen infected spleen, thymus or lymph node tissues were limited by the amount of cellular debris present. Tissue cultures when inoculated with homogenates were difficult to interpret due to residual cell membranes and organelles. Homogenates, however, were used to infect mice, rats, guinea pigs and chicken yolk sacs. Guinea pigs were found to harbor a low grade infection. A slight febrile response occurred in conjunction with lymph node involvement. Rickettsiae could be identified in lymph node impression slides. Serial passage of lymph node suspensions resulted in the progressive loss of the rickettsia. The infection was not as apparent in mice and rats and was lost after the second passage.

Immunosuppressants

The adrenal corticosteroid, cortisone acetate, was used to deplete small lymphocytes and diminish primary and secondary antibody responses. Cortisone was found to be most effective when given prior to the inoculation of the rickettsial suspension. Lymph node impression smears contained more rickettsiae than did those not treated with cortisone. The infection was lost in guinea pigs after the tenth serial passage.

Isolation of Rickettsiae in Yolk Sacs

Neorickettsia helminthoeca was found to grow in the yolk sac of six day old chicken eggs. After three blind serial passages rickettsia were observed in impression smears. Rickettsial infection resulted in arrested development and eventual death of the embryo on the fourth day post infection. Yolk sac membranes were found to harbor viable rickettsiae after freezing in liquid nitrogen. Rickettsiae were not present in embryos which had not been inoculated.

Mycoplasma Isolation

Mycoplasmas (pleuropneumonia-like organisms) were not isolated from organ suspensions of tissue culture fluids containing Neorickettsia helminthoeca. These results are not conclusive due to the limited isolation media utilized.

Tissue Cultures

Primary cultures of canine leucocytes were found to support the growth of Neorickettsia helminthoeca. The predominant cells of primary cultures were small, round mononuclear cells with little cytoplasm. The size of these cells increased after 48 hours in culture. It was not possible to categorize these cells into distinct types of leucocytes due to the presence of transitional forms. Primary cultures were dissociated on occasion (0.2% ethylenediaminetatraacetate in calcium magnesium-free phosphate buffered saline solution) and subcultured. In vitro passage of the organism was achieved by inoculation of normal monocyte cultures with fluids from infected cultures. Rickettsiae were detected in inoculated cultures after 48 hours of incubation. The rickettsiae multiplied during the subsequent 14 days of incubation and were always restricted to the cytoplasm. Infected monocyte-like cells were found to double or triple in size as the infection progressed. Rickettsiae were extruded from infected cells by microfibrillar structures protruding from the surface of the cell. The released rickettsiae were regularly extruded and dispersed slowly by Brownian movement, although rickettsial packets were occasionally observed which had adhered to the culture vessel. All of the cells in culture did not become infected at the same time. Cells with numerous rickettsiae were often found adjacent to other cells which did not contain

intracellular rickettsiae. As rickettsial multiplication proceeded more cells became infected so that by eight days post inoculation the majority were infected. When rickettsiae accumulated in considerable amounts within an infected cell, their multiplication stopped.

Primary cultures of chicken embryo fibroblasts, salmonid embryos and cultures of Samia cynthia did not support the growth of Neorickettsia helminthoeca. Slight growth was observed in chicken fibroblasts on two occasions, but the infection was not successfully transferred.

Canine kidney cells (CCL34MDCK) supported the growth of the rickettsiae although optimal growth did not occur. The cells could be infected by centrifuging the cells with infected material.

Canine sarcoma 503 cells and mouse lymphoblasts (CCL32MBIII) both supported the growth of Neorickettsia helminthoeca.

Fate of Inactivated Rickettsiae

Heat inactivated rickettsiae were found intracellularly after the cells had been exposed to them for one hour. Inactivated rickettsiae could not be found in inoculated cultures four hours after they were initially observed.

Inoculation of Dogs with Cultures

Dogs inoculated with fluids obtained from tissue cultures infected with Neorickettsia helminthoeca developed signs of salmon poisoning disease (Figure 5). Non-infected control dogs remained disease-free (Figure 5). The blood from infected dog D-6 was used to reisolate the organism in cell culture.

DISCUSSION

The present study was concerned with Neorickettsia helminthoeca: its relationship with the Elokomin fluke fever agent, its isolation and in vitro cultivation, its ultrastructural characteristics and the fulfillment of Koch's postulates as completely as possible for an obligate intracellular parasite.

Millemann and Knapp (1970) confirmed that canine hosts that recovered from salmon poisoning disease developed a lasting and solid immunity to the disease agent, but not to the fluke. Farrell (1964, 1968), however, found that salmon poisoning was a complex of two diseases, both carried by Nanophyetus salmincola. Results of this study suggest only Neorickettsia helminthoeca is involved in salmon poisoning in this area. Repeated experimental infections with metacercarial cysts elicit the same sharply peaked temperature curve seen with experimental infection of Neorickettsia helminthoeca isolates. The plateau-type temperature response characteristic of Elokomin fluke fever was never observed in animals infected with metacercariae. Animals that recover from infection of either agent are immune to challenge with the homologous agent. An animal which has recovered from one agent had no immunity when challenged with the other agent.

Staining reactions and pathology of infected cells and tissues agree with those reported by Cordy and Gorham (1950), Farrell (1966, 1968), Nyberg (1967) and Millemann and Knapp (1970).

The ultrastructural characterization of Neorickettsia helminthoeca was found to be typical of other rickettsiae described (Anderson et al., 1965; Higashi, 1968). Rickettsial cells were seen as circular profiles or rod-shaped (about 0.5μ wide and up to 0.7μ long). Some variation was found in the shape of the rickettsiae with some cells larger and less electron dense than the more numerous forms. Structural components were, however, always the same. Rickettsial cells were bounded by a cell wall, which surrounded the entire organism, and the underlying cytoplasmic membrane. Each of the membranes was a tri-layered structure and showed the unit membrane structure.

Time-lapse cinemicrography of infected and control cultures gave poor results. The cells were not flat and consequently out of focus. Rose chambers were found to be subject to changes in barometric pressure resulting in focus changes. The optical density of the rickettsiae was not optimal for continuous observation. Schaechter (1957) successfully recorded the growth of Rickettsia tsutsugamushi and Rickettsia rickettsia. These organisms were, however, 1.5μ long and more dense.

Cultures and tissues infected with Neorickettsia helminthoeca could be frozen in liquid nitrogen with recovery of viable rickettsiae in reduced numbers. The most effective method of storing Neorickettsia helminthoeca was in infected spleen tissue in Snyders I solution. Infective rickettsia could be obtained if the ampule was

thawed rapidly in a 37°C water bath.

Early attempts to isolate rickettsiae from experimentally infected dogs upon necropsy gave varied results. Spleen, thymus and lymph node tissues were used to establish primary cultures. Bacterial contamination of cultures was a problem due to rickettsial sensitivity to the concentrations of antibiotics normally employed to inhibit bacterial growth. Viral contaminants of cells were also observed. Rickettsiae, however, were observed in these infected cells on occasion. Interpretation of infected cells was difficult due to the residual cellular membranes and organelles present. Purification of cellular homogenates was complicated by the extracellular instability characteristic of most rickettsiae (Brezina, 1969; Brock, 1970; Elisberg, 1969; Millemann and Knapp, 1968; Moulder, 1962; Ormsbee, 1962 and 1969; Paretsky, 1968; Weiss, 1967 and 1968; and Wisseman, 1968).

Considerable effort was put forth to extend the experimental host range to animals other than canids. Dogs were costly to purchase and maintain. Various rodents were screened for susceptibility, but in all cases the infection was of a low grade and rapidly lost (Jackson et al., 1951; Philip, 1954; Philip et al., 1953). Treatment with corticosteroids (Caldwell, 1971; Piliero, 1970) did not appreciably increase the level of infection.

The discovery by Cox as described by Vinson (1958) that rickettsiae of the spotted fever group and typhus in cultures of chick embryo yolk sac tissues led to his inoculating the rickettsiae directly into the yolk sac of the developing chick embryo. The rickettsiae multiplied in the entodermal cells lining the yolk sac. Rickettsiae were thus easily obtained by this method in quantities sufficient for the production of practical volumes of vaccine and antigen and for studies of the biological properties of the rickettsiae. Neorickettsia helminthoeca harvested from infected cultures and tissues was found to grow in six day old chicken yolk sac embryos and to depress the development of the embryos. Yolk sac membranes were harvested and passed to new egg yolk sacs. By the eighth passage of the rickettsiae 100% mortality of the embryos was observed 96 hours post inoculation. Rickettsiae were observed in yolk sac smears prepared from these embryos although in low concentration. Control embryos were not infected and rickettsiae were not observed in yolk sac smears. The rickettsiae in infected yolk sac cells were found to be viable after storage in liquid nitrogen. Stoenner et al. (1962) found that yolk sacs infected with Rickettsia rickettsia or Rickettsia conorii which died after 100 hours regularly contained toxin. Neorickettsia helminthoeca was not found to grow optimally in embryonated chicken eggs under the conditions tested, although death of infected embryos was observed.

Mycoplasmas were not found associated with salmon poisoning disease. This conclusion is based on the limited media tested. This area merits further investigation using a wide variety of isolation procedures (Stanbridge, 1971).

In animal pathology, the most frequently studied host-parasite interaction is that of a multicellular host with a unicellular parasite. The host is a collection of many different interrelated populations of cells, and the parasite, although unicellular, is distributed in or among different host-cell populations. The phenomenon of intracellular parasitism offers a way around some of the difficulties encountered in working with whole animals. The complex relationship between intact host and parasite can be reduced to host cell and parasite by using uniform populations of single susceptible cells as the host (Moulder, 1962, 1969).

Neorickettsia helminthoeca was found to grow in cell cultures of canine leucocytes, sarcoma cells and mouse lymphoblasts. The applications of quantitative methods to multiplication of Neorickettsia helminthoeca were restricted by the inability of the organisms to grow in easily accessible laboratory animals which could be used to quantify amounts of viable rickettsiae present, i.e., LD_{50} (Bozeman et al., 1956) or sufficiently well in chicken embryo yolk sacs to calculate $YSLD_{50}$/ml (Weinberg et al., 1969).

Rickettsiae which were heated at 56°C for one hour were inactivated. They were found intracellularly one hour post exposure but not after four hours. This observation precludes the assumption that rickettsiae entered the cell by means of a penetrating mechanism. Inactivated rickettsiae are thought (Vinson, 1958) to have increased susceptibility to the destructive action of the cell. The actual entry of rickettsiae into cells has been studied by Cohn et al. (1959) who concluded that both rickettsia and host cell must be viable and that divalent cations and proteins must be present in the medium for entry to occur.

The in vitro cultivation of Neorickettsia helminthoeca allowed the fulfillment of Koch's postulates as completely as possible for an organism which is an obligate intracellular parasite. Neorickettsia helminthoeca was found in all cases of the disease. Neorickettsia helminthoeca was isolated from the blood of infected dogs and grown in cell cultures. The pure isolated culture reproduced the disease when inoculated into a susceptible dog. The organism was observed in and recovered from the inoculated dog. Since rickettsiae must be grown in living cells positive proof of purity was not possible. The infection resulting from inoculation of cultured rickettsiae was similar to natural infection except for an increased incubation period.

The cultivation of Neorickettsia helminthoeca in cell culture of canine monocytes, sarcoma cells or mouse lymphoblasts offers a

tool to elucidate the mechanisms by which the infective process is accomplished. A cell culture, however, is a dynamic system with its own inherent complexities (Vinson, 1958). Since it is an artificial model, phenomena described as occurring in a cell culture system cannot be directly extrapolated to corresponding phenomena assumed to occur in the intact host. The study of Neorickettsia helminthoeca in cell systems is of value in extending our knowledge of the biology of this organism. The essential nature of obligate parasitism, however, remains an enigma. Cultivation of rickettsiae in defined non-cellular media is a prerequisite for an understanding and appreciation of obligate parasitism.

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