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To

AGENCY FOR INTERNATIONAL DEVELOPMENT

And

THE ROCKEFELLER FOUNDATION

Texas A&M University

College of Veterinary Medicine

Institute of Tropical Veterinary Medicine

PROGRESS REPORT

June, 1969 - June, 1970

To

AGENCY FOR INTERNATIONAL DEVELOPMENT

And

THE ROCKEFELLER FOUNDATION

From

Texas A&M University

College of Veterinary Medicine

Institute of Tropical Veterinary Medicine

Fred D. Maurer, Director

Concerning work done at:

Laboratorio Investigaciones Medicas Veterinarias
Bogota, Colombia

College of Veterinary Medicine
Texas A&M University
College Station, Texas

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(and collaborators)

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SUMMARY - AID ANNUAL REPORT

July, 1970

The establishment of the Institute of Tropical Veterinary Medicine at Texas A&M University in January, 1967, and its subsequent support by the Rockefeller Foundation and the Agency for International Development, has put in motion plans to develop greater competency, and understanding of animal disease problems in the tropics.

The professional staff has increased from a single full time employee in 1967 to 3½ full time staff positions, not including the Institute Director, Dr. F. D. Maurer, who also serves as Associate Dean for Research in the College of Veterinary Medicine. During this same time a total of 4 Technicians, 2 secretaries and 2 farm laborers have been employed plus student help. In addition there are 4 Master of Science and one Doctor of Philosophy candidates actively pursuing studies of tropical veterinary medicine in the discipline of their choice.

The arthropod born hemotropic diseases were initially chosen as a major area of study in recognition of the critical need for information and techniques for the control of these diseases in the tropics. The Institute's research program began in July, 1967, at Bogota, Colombia as a cooperative effort with the Instituto Colombiano Agropecuario (ICA). In December, 1968, following an increase in staff, a research program was started on the Texas A&M campus. Presently, research is actively underway at both locations. In 1969 Texas A&M staff members at Bogota became affiliated with CIAT, recently established and based at Cali, Colombia.

Research conducted on anaplasmosis, babesiosis, and to a lesser degree trypanosomiasis has resulted in numerous scientific publications. Of greater significance and interest to the livestock producer in the tropics have been the development of techniques which should form the basis of future control programs. These findings have not been developed without setbacks, and reversals, but a method or an approach appears to be taking shape which when implemented should dramatically reduce losses from anaplasmosis and babesiosis. These techniques while hopefully proving useful, should realistically be considered a first step toward eventual complete control.

A limited survey of cattle herds located in various climatic and geographic zones of Colombia revealed that anaplasmosis was endemic in those areas where the altitude was 4,000 feet or less. At 8,000 ft. and above the disease was not found except in animals having been brought into the area. The incidence and severity of anaplasmosis appeared to increase as the altitude decreased. This correlation between altitude and disease incidence would probably apply to other tropical countries if an adjustment is made for latitude.

The problems of anaplasmosis and babesiosis were found to be most severe at the ICA station (Turipana) near Monteria on the north coast of Colombia. For this reason Turipana was chosen as the location of field trials attempting to suppress the often devistating effects of anaplasmosis and babesiosis in introduced cattle. A live vaccine using at attenuated A. marginale strain and a killed vaccine, both developed in the U.S., were unsuccessful in producing the desired protection in calves being moved to Turipana. Under less severe conditions, particularly in the U.S., these vaccines have been shown to be of value.

A method premunization using virulent organisms proved successful. On 2 occasions experiments consisting of the intentional infection of susceptible calves with fully virulent Anaplasma produced solidly immune animals. The experimental infection was moderated by the use of drugs given at the proper time. Both the oxytetracyclines and a new experimental drug designated 356C61 (a dithiosemicarbazone developed by Burroughs Wellcome Laboratories) are useful.

A similar premunizing infection with Babesia proved nearly as successful. In this instance 35% of the non-premunized control calves died and all became acutely ill requiring treatment. The premunized calves on the other hand were slower in developing evidence of babesiosis, and when it did occur were much less severely effected. None of the vaccinated calves died of babesiosis. It has been found that babesiosis due to a virulent Babesia can be successfully controlled with either Ganaseg, or a new experimental drug designated 4A65 (Burroughs Wellcome Laboratories).

These experimental trials will provide the basis of a control program soon to be tried in Colombia. Even though it is anticipated that these measures will in part succeed, safer, and more effective measures are called for.

Recent experimental results have contributed significantly to our understanding of these diseases and by so doing are providing the basis on which more effective control programs can be implemented for the future. A few of the more significant findings are listed:

1.) The drug 4A65 has shown marked prophylactic value against Babesia for up to 46 days after treatment.

2.) Killed adjuvant vaccines against both anaplasmosis and babesiosis have been prepared, and are being tried. Vaccines against babesiosis show promise.

3.) Sterile immunity has resulted in mice following a B. rodhaini infection and treatment. A follow up on this finding might lead to a new approach to immunity to babesiosis.

4.) Drug trials with 4A65 have shown it superior to Ganaseg in the suppression of B. rodhaini, but somewhat toxic to calves at 3 mg/kg level.

5.) A complement-fixing antigen has been prepared from Babesia infected blood forming the basis of a successful serologic test for the detection of babesiosis.

6.) Other Babesia serological tests such as double gel diffusion, immuno-electrophoresis, and latex particle agglutination have been developed which offer greater sensitivity and a greater potential for the eventual recognition of serologic types and sub-types.

7.) Transmission studies have been made with the tick Boophilus annulatus. B. annulatus transmits both anaplasmosis and babesiosis to cattle. Transmission of anaplasmosis and babesiosis by B. annulatus to white tailed deer (Odocoileus virginianus) did not occur.

8.) Detailed studies of the biochemic and pathologic changes associated with anaplasmosis, babesiosis, and trypanosomiasis, have been or will be made. These studies while very basic in nature have never been conducted in the debth planned.

9.) The drug 356C61 while highly effective in removing A. marginale parasitemia has not been shown capable of entirely removing the infection at sub-toxic levels.

10.) Efforts to experimentally adapt A. marginale to goats have been unsuccessful. Attempts to establish a non-bovine model are now being made with sheep.

11.) Research with T. vivax and T. theileri have been confined mainly to distribution and pathogenesis studies. T. theileri has been found in both Colombia and Texas.

12.) Boophilus microplus, Amblyoma cajennense and Dermacentor nitens were identified on animals infected with anaplasmosis and babesiosis in various parts of Colombia.

From this study, very interesting observations can be made. Boophilus microplus were present in the enzootic areas of babesiosis and anaplasmosis. This confirms previously published reports that this vector was involved in the eqizootiology of infection of Babesia spp. in Colombia. However, the fact that Dermacentor nitens were found on animals known to be infected with babesiosis and anaplasmosis is a very interesting finding and the significance of this is not evident. It is well known that Dermacentor nitens has as its host the horse, and it has been reported as a vector of equine babesiosis in the United States. The question remains is the bovine an accident host of Dermacentor nitens and, if not what is the role of this tick in the transmission of bovine babesiosis? These questions must be answered by additional experimental work to clarify this relationship before a control program of tick vectors can be designed.

The following hemotropic pathogens are being maintained by the Institute staff, at Texas and Colombia.

Anaplasma marginale (Texas virulent)

A. marginale (Llanos strain)

A. marginale (Attenuated strain)

Babesia bigemina (bovine)

B. argentina (bovine)

B. major (bovine)

B. rodhaini (mice)

B. cervi (deer)

Trypanosoma theileri (bovine)

T. vivax (bovine)

Theileria cervi (deer)

The many and varied facets of research results on the hemotropic diseases are constantly adding to our knowledge and filling in the voids concerning our understanding of these complex and costly diseases. An integral part of our program is the field application of these findings which will hopefully lead to a practical, and effective control approach to these disease problems in the tropics.

In addition to research activities the program of graduate training is proving very successful. Dr. C. A. Carson received a Masters degree at Texas A&M based in part on his research in Colombia on anaplasmosis. Drs. Jones and Daley are in the final phases of their research in Bogota, and will be submitting a research thesis to support their application for the Masters Degree. In addition there are 3 graduate students at Texas A&M who have essentially completed their course work, and are now preparing to depart for Colombia to initiate their research programs. Drs. Thompson and Corrier will be pursuing a Masters degree in microbiology and pathology respectively. Dr. Bishop is working for a Ph.D. degree in microbiology. We anticipate 3 additional graduate students beginning this next fall.

Section II

COLOMBIA ANNUAL REPORT OF HEMOTROPIC DISEASE RESEARCH AND TRAINING

MARCH, 1969 TO JULY, 1970

TEXAS A&M GROUP IN COLOMBIA

INSTITUTO COLOMBIANO AGROPECUARIO

LABORATORIO DE INVESTIGACIONES MEDICAS VETERINARIAS

CIUDAD UNIVERSITARIA, BOGOTA, D. E.

COLOMBIA

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PREFACE AND ACKNOWLEDGEMENTS

The majority of the research was executed at the L. I. M. V. - ICA laboratories, but certain projects were conducted at the Palmira and Turipaná - ICA experiment stations. Acknowledgement is gratefully given to ICA and its professional staff for providing the facilities and administrative support in helping to fulfill the common objectives.

Acknowledgement is also given to Dr. R. K. Waugh, Dr. U. J. Grant, Dr. E. D. Roberts, Dr. N. S. Raun, Dr. Jaime Estupiñán and Dr. Guillermo Mateus for the guidance and professional administrative support that has been so beneficial to the Texas group.

Acknowledgement of the assistance given by the Rockefeller Foundation, United States Agency for International Development, Centro Internacional de Agricultura Tropical, Texas A&M University and Instituto Colombiano Agropecuario is also gratefully given.

This annual report is intended to serve as a guide of the progress made in hemotropic disease research and as a reference of future research to be performed in Colombia. It is understood that no part of this report is to be published or otherwise communicated except by permission of the authors.

I. INTRODUCTION

In general, the Texas Group has functioned as part of the National Program of Veterinary Parasitology under the direction of Dr. Guillermo Mateus. Since the Texas Group is a part of this program, it has close liaison with Entomology and associated fields which are closely related to the field of hemotropic diseases. This association has augmented the efforts of both the Texas Group and the National Program of Parasitology, by this it is meant that the Texas Group has cooperated in the training of some ICA personnel in the field of parasitology while ICA has helped the Texas Group by identifying insect specimens and furnishing laboratory supplies and equipment.

From all appearances, the relation between the Texas Group and ICA personnel is excellent and the working situation is extremely good. It appears that the program in hemoprotozoology has meshed with the National Program of Parasitology in all instances and is completely acceptable to both entities. In general, research has been primarily performed in the areas of disease prevalence, diagnosis and vaccines with some basic work being done on the effects of the various organisms in the bovine host. Several serological and immunological methods have been developed for the first time in Colombia and it is hoped that these methods can be used to fulfill the common goal of control of these important diseases caused by hemotropic organisms.

Dr. L. G. Adams and Dr. R. A. Todorovic have worked together continously during the last year to complete as many of the projects related to

anaplasmosis, babesiosis and trypanosomiasis. They were joined by Dr. Floyd M. Jones and Dr. Charles A. Daley in September, 1969. Drs. Jones and Daley are completing the thesis portion of their Masters degree in Colombia and their respective reports are included in the research portion of this report.

The Texas Group in Colombia is technically supported by 3 laboratory technicians and one bilingual secretary.

II. ANAPLASMOSIS

A. Anaplasmosis Projects in Progress

1. A Study of the Pathogenesis of Anaplasmosis in Intact Calves: Including Serological, Clinical Pathological, Histopathological and Immuno-fluorescent Methods of Study

Sufficient investigation into the host-parasite interaction has not been thoroughly studied particularly as related to the site of proliferation of Anaplasma marginale within the intact calf.

Objectives:

1. To study the clinical pathological alterations of anaplasmosis in comparison with the sequential pathological lesions produced by Anaplasma marginale.
2. To investigate the possibility of localization and/or proliferation of Anaplasma marginale within host tossies and to compare this with serological reactions.

3. To attempt to correlate the localization of the organisms with the sequence of pathological lesions, clinical pathological alterations and serological reactions.

Materials and Methods:

Twelve 4-month old, male, Holstein calves were screened for the hemoprotozoal disease by inoculating pooled blood samples into a splenectomized calf, by the complement fixation test for anaplasmosis and babesiosis and by preparation of wet mounts. The calves were examined weekly for 12 weeks prior to the inoculation of A. marginale for packed cell volume, percentage parastemia and general state of health. One week prior to the inoculation of A. marginale, 3 control samples at 2-day intervals were taken for determination of the following parameters: packed cell volume, hemoglobin content, bone marrow biopsy, total serum protein levels, blood glucose content, body temperature, complement fixation titers, blood smears, serum glutamic-oxalo acetic transaminase concentrations, sorbitol dehydrogenase levels, direct, indirect and total bilirubin values, total leukocytes, differential leukocytes and serum protein electrophoresis.

On day 0, the calves were inoculated with a Colombian Llanos isolate of A. marginale, maintained in a carrier calf, at a dosage of 5 ml. subcutaneously. All of the above mentioned parameters were performed on all of the remaining calves every

other day except for bone marrow biopsies and body temperatures. Bone marrow biopsies were performed on 3 different calves every 2 days and body temperatures were determined daily.

Thereafter, one calf was euthanized every 2 days for 24 days. Two complete sets of tissues were collected. One set of tissues was collected in buffered 10% formalin and the other set was quickly frozen with solid CO_2 onto wooden blocks with Cryoform and stored at -65°C.

All tissues were parafin processed, sectioned at 6 mu. and stained with hematoxylin and eosin. Frozen sections of the collected tissues were made on a Cryostat microtome and indirectly stained with fluorescein-labeled antibodies for A. marginale for 30 minutes in a humid 37°C chamber, rinsed 3 times in phosphate buffered saline pH 9 and cover-slipped with carbonate-bicarbonate glycerol solution at pH 9. Fluorescent antibody conjugates were prepared against bovine gamma-globulin in the following manner: Six adult, New Zealand rabbits were inoculated with bovine gamma-globulin prepared by the precipitation of all serum proteins except gamma and beta globulins with ethodin at 4°C, which were decanted. The ethodin was removed by absorption with activated charcoal and centrifugation. The purified, bovine gamma globulin was then concentrated by lyophilization, reconstituted in buffered physiological saline and injected into the rabbits intramuscularly 4 times at weekly intervals. Two weeks after the final injection, the rabbits were exsanguinated and serum was

collected. The rabbit serum containing the anti-bovine, gamma-globulin was precipitated as described previously and the purified rabbit anti-bovine gamma-globulin was concentrated by dialysis against polyvinyl-pyrrolidone to the original volume. The globulin fraction was dialysed against carbonate-bicarbonate buffer 0.5 M pH 9 in physiological saline. The protein content of the solution was determined at 280 mu. in a Beckman spectrophotometer Model D B. Fluorescein-isothiocynate was added at a rate of 0.05 mg. per each mg. of protein to the surface of the globulin fraction with continual stirring for 12 hours at 4°C. Excess fluorescein dye was removed by dual passage through 2 G-25 cooled Sephadex columns, and the rabbit anti-bovine gamma globulin conjugate was collected in 5 ml. fractions, lyophilized and stored at -20°C. The reconstituted conjugate was used to stain the anaplasmosis specific bovine gamma globulin which was attached to A. marginale organisms in the blood smears and frozen tissue sections. The stained specimens were examined with a Leitz Ortholux ultra-violet microscope with a HBO-200 lamp and a BG 12 excitor filter and a 570 barrier filter.

Results:

The results of the serological clinical, clinical pathological examinations are recorded in the following: Figures 1, 2, 3, 4, 5 and 6.

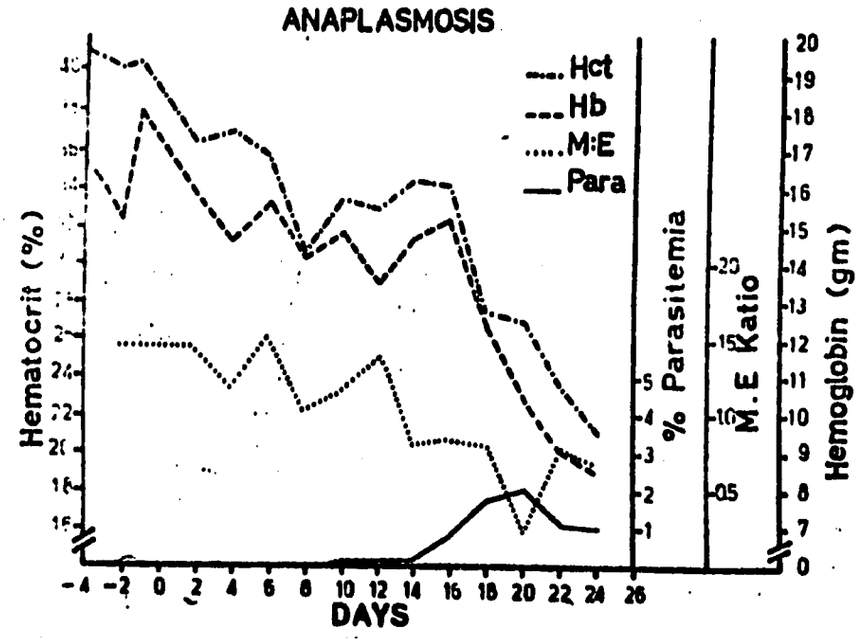

Fig. 1. The packed cell volume, hemoglobin content and percentage parasitemia of peripheral blood are shown in relation to one another and time in days. In addition, the myeloid: erythroid ratio of the bone marrow is depicted.

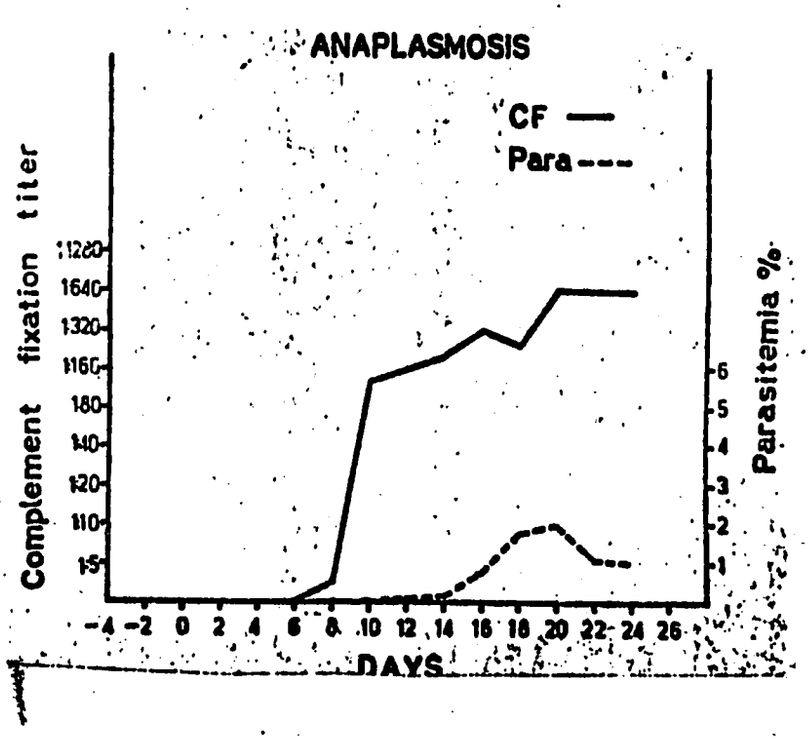

Fig. 2. The complement fixation titer of anaplasmosis is compared to the incidence of parasitemia in peripheral blood.

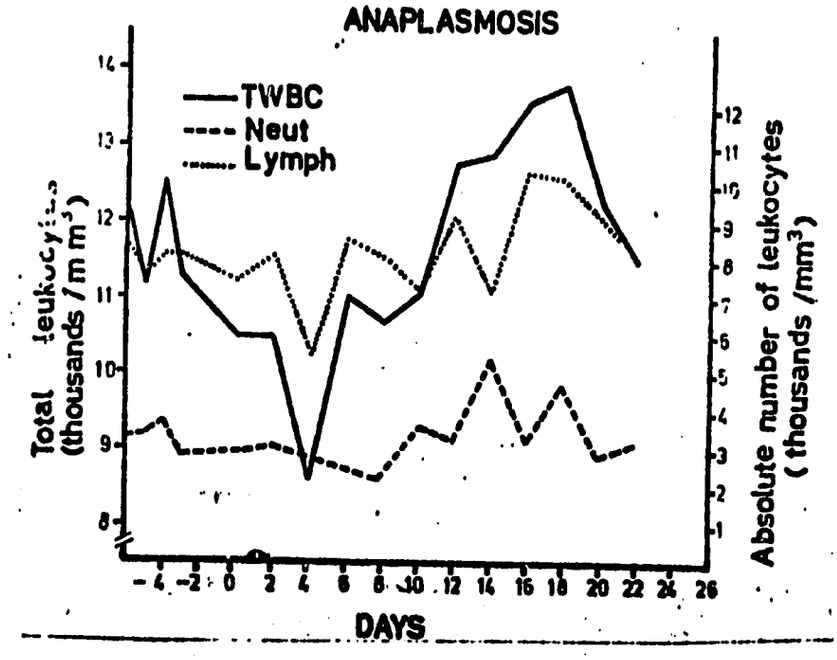

Fig. 3. Total leukocytes and absolute numbers of neutrophils and lymphocytes in peripheral blood are compared to one another and time.

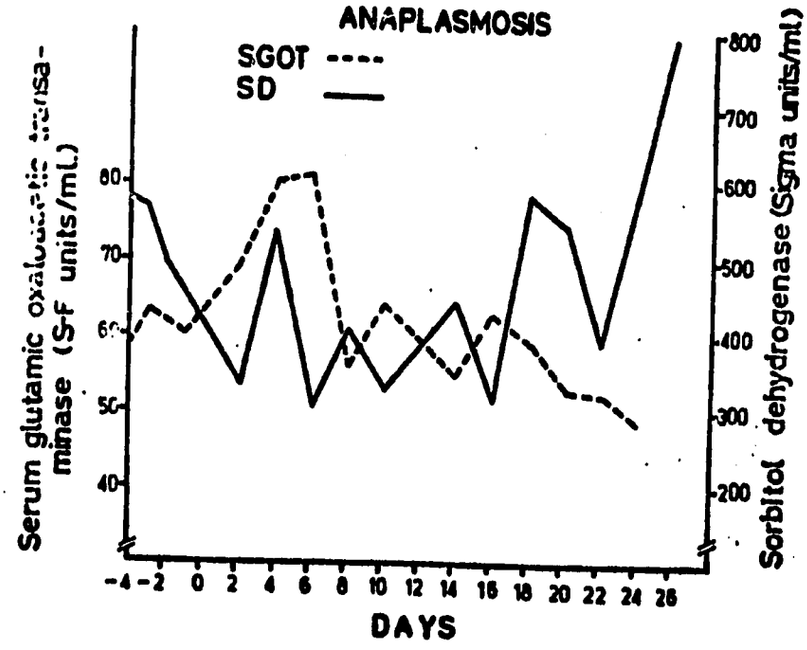

Fig. 4, Serum glutamic-oxaloacetic acid transaminase concentrations and sorbitol dehydrogenase levels in the peripheral blood of anaplasmosis infected calves are shown in relation to time.

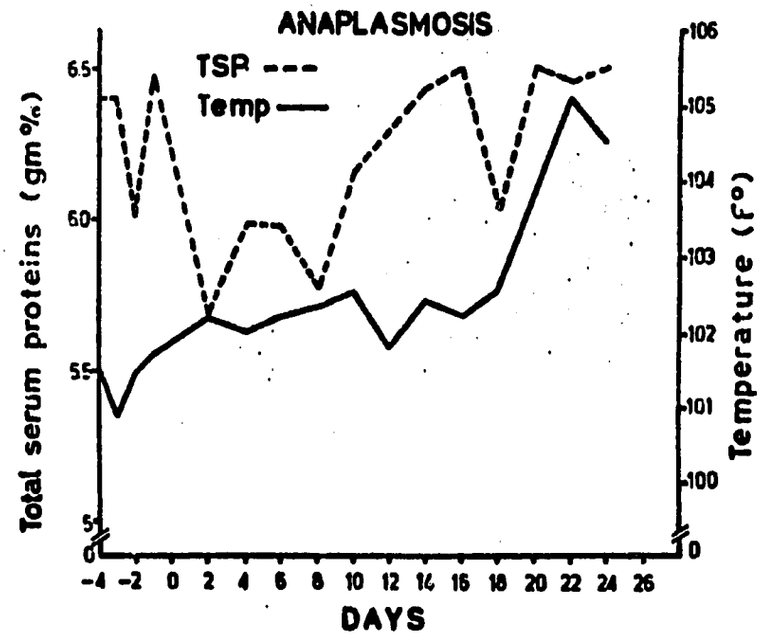

Fig. 5. Total serum proteins are compared to the variations in body temperature.

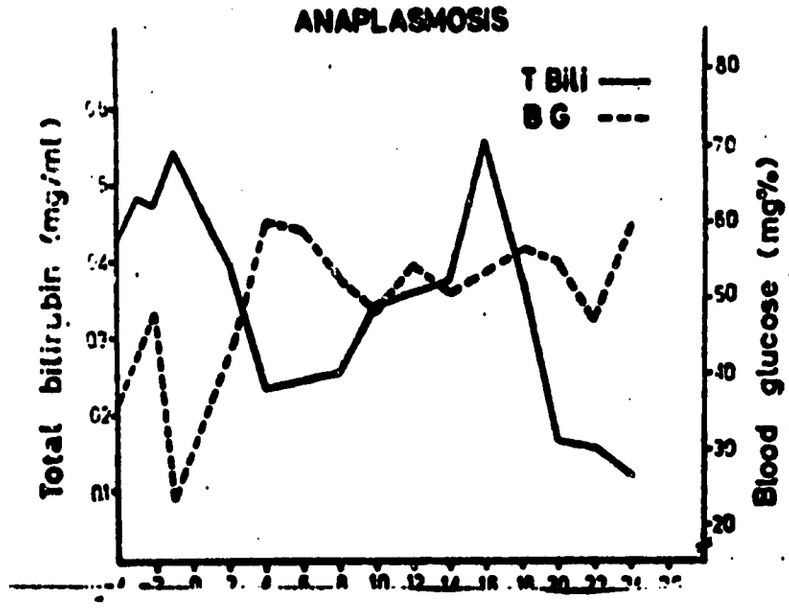

Fig. 6. Total bilirubin and blood glucose concentrations of blood are compared in relation to time.

Results of the histopathological and immuno-fluorescent studies are not completely terminated, therefore, no analysis can be made at this time, but there is a general reaction by the reticulo-endothelial system which is particularly evident in the spleen and lymph nodes.

Discussion:

Because the experiment is not completely finished, no specific conclusions can be made, however some very interesting observations can be discussed. Four days after challenge with A. marginale the total leukocytes and lymphocytes were significantly decreased perhaps indicating an effect upon their production, yet the neutrophils were not affected. It is interesting to note that the total serum proteins and blood glucose decreased while the total bilirubin values began a gradual, upward increased perhaps indicating the onset of hemolysis at low levels.

On day 6 PI, the complement fixation test became positive in some of the calves preceding the parasitemia by 4 days. From day 10 PI to day 16 PI the parasitemia, total leukocytes, lymphocytes, neutrophils, total serum proteins and the complement fixation antibody titer increased as the myeloid: erythroid ratio gradually decreased indicating a general humoral reaction to the organisms. Day 18 PI through day 20 PI appeared to be the critical phase of the disease, because the hematocrit, hemoglobin content, began to decrease as the myeloid: erythroid ratio reached

its lowest value and the parasitemia, total leukocytes, lymphocytes, total serum proteins and complement fixation antibody titer reached their respective highest levels, indicating that the animal is in a severe state of disequilibrium, but is trying to combat the organism by many methods. In the last 4 days, day 24 PI the packed cell volume and hemoglobin content continue to decrease as the temperature increases. The bone marrow begins to respond and the leukocytes, CF titer, total serum proteins level off as the parasitemia decreases.

2. Control of Anaplasmosis and Babesiosis Simultaneously in Young Cattle.

a. Introduction

A great deal of laboratory and field investigation has been performed on methods of control for anaplasmosis and babesiosis as separate disease entities. Because these diseases rarely occur singularly, especially in Colombia, further investigation of simultaneous control under actual field conditions should be attempted. Three different geographical areas and elevations will be selected for this investigation.

b. Objectives:

A. To evaluate and monitor anaplasmosis and babesiosis vaccination and premunization procedures under actual field conditions.

B. To evaluate tick population control measures with certain chemicals under varied conditions.

C. To evaluate the efficacy of two recently developed compounds in anaplasmosis and babesiosis premunization.

c. Status of the Question

The hematozoan diseases of anaplasmosis and babesiosis can be found worldwide and are of greater economic importance in cattle than in any other species of animal. In the warm climates and in tropical countries, these two diseases are responsible for a considerable and productive loss. Although the indigenous cattle carry the infection without clinical signs, introduced animals are usually highly susceptible to attack. Carrier animals, in spite of their inapparent infection, often suffer a decided decrease in meat and milk production. Also, they are capable of infecting ticks and possibly other vectors thus propagating the disease cycle.

In order to develop the present status of anaplasmosis and babesiosis control, these diseases, though similar in many ways, will be discussed separately.

Anaplasmosis is an infectious hematozoan disease of cattle cattle manifested by a rapidly progressive anemia with a syndrome divided into a prepatent period, a period of increasing anemia, maximal anemia, and a period of convalescence.

The research developments in the field of anaplasmosis began

in 1893 when Smith and Kilbourne studying the erythrocytes of infected cattle, observed "intracorpuscular bodies." They considered these coccus-like bodies to be a stage of the life cycle of Babesia bigemina. Theiler and associates in 1910 started a series of studies on this organism which they correctly differentiated from Babesia organisms and named Anaplasma marginale. Studies of this disease continued over the years and in 1951 DeRoberts and Epstein revealed that the "chromatin body" described by Theiler in 1910 was not a compact single entity but rather a number of smaller sub-units.

Other significant findings have been made since the early 1950's. Two such developments were the discovery that the tetracycline compounds inhibited the Anaplasma organisms, and the development of the complement fixation (CF) test for diagnosis of anaplasmosis. The CF test was the first reliable test for detecting carrier animals and greatly enhanced the study of the serological responses of an animal to the infecting Anaplasma organism.

The specific staining of Anaplasma with fluorescein labeled antibody identified it as the causative agent to which the host produced specific antibodies. Ristic in 1967 initiated a series of studies which showed that the sub-unit of the marginal body (initial body) was the true organism, and that its ability to invade mature erythrocytes and multiply in them by binary fission

led to the formation of the marginal body. The development of the capillary tube agglutination (CA) test in 1962 provided a relatively simple and effective means of diagnosing anaplasmosis.

The transmission of anaplasmosis can occur by both biological and mechanical means. In biological transmission, the causal organism persists through various stages of the vector. At least twenty species of ticks are capable of biologically transmitting anaplasmosis under experimental conditions. Most cattle ticks can also act as mechanical vectors. Experimental and epidemiological evidence incriminates horsefile as a significant mechanical insect vector. Many other insects and mosquitoes are potential natural vectors and have been incriminated in experimental studies. In addition to mechanical transmission by insects, transmission by syringe needles and surgical instruments is possible and is reported to have occurred.

The knowledge of mechanisms of immunity in bovine anaplasmosis is far from complete. Nevertheless, some basic findings concerning immunopathology have been made during the last twenty years. The principal states of immunity (resistance) which are encountered in infections with A. marginale are (1) innate species resistance, (2) natural resistance, (3) idiopathic resistance, and (4) acquired resistance or immunity. This project is mainly concerned with acquired immunity since this includes the vaccination and premunization procedures for the control of this disease.

Acquired immunity in anaplasmosis can be defined as the immune state of an animal resulting from exposure to Anaplasma

antigens. If Anaplasma organisms are used to infect an animal and specific chemotherapy is administered shortly thereafter to destroy the infectious organisms, the residual immunity which persists after the hemoparasites have been eliminated can be called "sterile immunity with previous infection." The second type of sterile immunity results from exposing a susceptible, Anaplasma-free animal to killed organisms or their antigens and can be called "sterile immunity without previous infection." The final type of acquired immunity depends on the persistance of a latent infection in animals which survive the initial infection. This acquired immunity to reinfection can be termed "non-sterile immunity, premunition or co-infectious immunity." There are many reports of attempts to provide an immune response in susceptible cattle by inoculating them with antigens of killed Anaplasma preparations. Some of these vaccine are currently on the commercial market. Although vaccinated animals show some resistance, all of them can become infected following challenge. From a practical viewpoint, a sterile immunity against A. marginale has not been produced by these agents.

Ristic suggests that a lasting resistance to this disease can be established by providing for the constant maintenance of the immunopathological process associated with sub-clinical infections. This could best be accomplished by a live attenuated Anaplasma vaccine or premunization with virulent A. marginale organisms in susceptible animals. This proposed project will

evaluate both of these procedures simultaneously with babesiosis premunization and vaccination.

Babesiosis, a tick-transmitted diseases of animals, is associated with the appearance of the specific infecting protozoa in the erythrocytes. It occurs in many animal species, but this study will be limited to Babesia bigemina and Babesia argentina which occur in cattle in Colombia. Bovine babesiosis is highly pathogenic and may cause high mortality in susceptible cattle. These blood parasites cause destruction of the red blood cells with a release of the vascular network of various organs by parasitized cells, cell debris and free parasites may cause an accumulation of toxic metabolic products, anoxia and possible breakdown of the blood capillaries and organic vessels.

The history of babesiosis dates from the discovery of Babesia bovis by Babes in 1888. Smith and Kilbourne in 1893 recognized by B. bigemina as the cause of Texas fever and proved that it was vectored by the cattle tick Boophilus annulatus. Lignieres in 1901 discovered B. argentina and incriminated this organism in bovine babesiosis. Both of the organisms are transmitted by the ixoid tick Boophilus microplus in South America. According to an unpublished report by Todorovic in 1969, both B. bigemina and B. argentina pass from the infected adult female tick through the eggs to the larval tick hatching from the eggs on the

ground. B. argentina is transmitted to cattle by the larvae of Boophilus microplus, whereas B. bigemina is transmitted by the nymphs and adult ticks and not by the larvae. Multiplication of the organism in the erythrocytes is usually by budding, giving rise to two to four daughter cells depending on the species. They do not form pigment from the host cell hemoglobin.

Most problems in immunity to babesiosis in cattle have been defined since the early studies of Smith and Kilbourne. The principal states of immunity to babesiosis in cattle closely parallel those of anaplasmosis. This project will be largely limited to acquired immunity.

It has been shown that B. bigemina and B. argentina in the absence of reinfection usually persist for 10-12 months and that severe relapses may occur at varying intervals within this period. Cattle which recover from clinically evident babesiosis develop a non-sterile immunity which may last for 1-12 months. However, it should be noted that the duration of this "immune" state varies considerably both with the species of Babesia and with individual animals. Many immunological differences demonstrated in strains under laboratory conditions also exist in the field, and the transfer of animals from one enzootic area to another may be followed by a new attack.

Most of the resistance produced by an individual animal

to this disease is the non-sterile immunity. There is some evidence in some animals of a graduation from a state of "premunition" to a state of sterile immunity. Riek and co-workers have shown that a tick-transmitted B. argentina infection usually persists for at least ten months, and that infections with B. bigemina may persist for at least 9-10 months. They also report that the duration of sterile immunity with B. argentina has not yet been determined. A non-sterile immunity was usually developed in cattle with a latent infection, and with B. argentina they report that one animal has shown evidence of a sterile immunity.

In a study of immunity to babesiosis, it becomes apparent that the state of equilibrium which develops between the host animal and the protozoan parasite is not permanent. The parasite will ultimately be eliminated in the absence of continued infection by ticks, and the animal may become susceptible to a clinical attack if reinfection occurs after a certain lapse of time.

In a report on bovine babesiosis in Australia, Todorovic (unpublished) quotes Mahoney and Hoyte as stating that outbreaks of Babesia may be seen under the following circumstances:

(1) When susceptible cattle from clean countries are taken into, or allowed to pass through tick-infested (Boophilus microplus) countries.

(2) Where the presence of ticks (B. microplus) is variable.

(3) When the tick (B. microplus) population has been reduced to a very small proportion by dipping.

(4) When cattle are transferred from a clean part of a property to a tick-infested part of the same property.

(5) When conditions are specially favorable to tick life.

(6) When cattle are removed from one tick-infested (B. microplus) area to another.

(7) Following the passage of tick-infested (B. microplus) cattle through a property previously clean or only lightly tick-infested.

(8) When carriers of Babesia infection suffer a relapse.

As Babesia are only transmitted by ticks, prevention of infection depends on the elimination of this vector. Obviously this is very difficult for some countries, but dipping and spraying has worked well in certain areas and this practice has widespread use.

In most enzootic areas, prevention of babesiosis has given way to the control of this disease. Most of the killed and attenuated vaccines are of recent development, and more research is going on in this field now. At the present time, control of infection may be carried out by the premunization of young or introduced stock and this procedure is also used to prevent outbreaks in marginal areas where tick infestation varies according to climatic conditions. This premunization or co-infectious immunity is accomplished by the injection of

infected blood and controlling the subsequent infection with one of the babesiacidal drugs. The complexity of the interaction of the host, the blood parasite and the tick vector suggests that more effective methods of prophylactic immunization are needed.

It logically follows that more effective control of anaplasmosis and babesiosis would help increase a country's protein food supply.

d. Materials and Methods:

This research project was conducted in three privately own dairy farms in Colombia in the Valle del Cauca (Palmira area), in the Upper Magdalena Valley (Girardot area) on the North Atlantic Coast (Montería-Cereté area).

Thirty animals were selected by age, sex and breed. Two to three-month old calves of the Holstein, Zebu and Lucerna breeds were used in this project. Both Heifer and male calves were in this experiment.

In order to keep the experiment on a practical basis, management practices, such as feeding and ration type were conducted by the dairy farmer as he chose.

Insecticides used for controlling the vector population depended upon what was available on the local market and what was in current use. Some preparation currently in use include toxaphene, neguvon and Asuntol (Bayer).

The research project consisted of 3 groups of 10 intact

calves on each dairy farm. Pre-inoculation blood samples were collected for the determination of prior exposure to Anaplasma and Babesia organisms. Every effort made to select test calves that are free from signs of or exposure to other disease conditions.

Group A (10 intact calves) was premunized with a standard inoculum from a carrier calf of the Llanos isolate of A. marginale and a standard inoculum of the Llanos isolate of mixed B. bigemina and B. argentina from a splenectomized carrier calf. Since a large dose of inoculum has been found unnecessary to produce these diseases, a 5 ml. subcutaneous injection of mixed Anaplasma and Babesia organisms was administered.

Group B (10 intact calves) was vaccinated with an attenuated isolate of A. marginale and a killed vaccine prepared from B. bigemina and B. argentina.

Group C (5 intact calves) served as an untreated control.

A survey of the literature indicates that many compounds have been used in the treatment and premunization against anaplasmosis and babesiosis. Two recently developed compounds will be used in the premunization of calves for this experiment.

Calves which are premunized against A. marginale were treated on day 5 after inoculation with Burroughs-Wellcome compound 356-C-61 (dithiosemicarbazone) at a dosage of 5 mg/kg of body weight. Animals premunized with Babesia spp. were treated on day 5 after inoculation with Burroughs-Wellcome compound 4A65

(3,3'-Bis-(2-imidazolin-2-yl) carbanilide dihydro chloride at a dosage of 1 mg/kg of body weight.

Blood samples for this experiment were collected at 7-day intervals. Blood smears were prepated according to the thick blood film technique developed by Mahoney and Saal. Packed cell volumes were determined by the micro haematocrit method as outlined by Schalm and complement-fixation tests were performed on the collected serums as recorded in the standard USDA procedure with modifications.

The immune response in cattle successfully challenged by Anaplasma and Babesia organisms is characterized by the production of serologically detectable antibodies that can be demonstrated by the CF and the CA tests. Also, the anemia produced during premunization is apparently associated with the autoimmune process and contributes to the resistant state, The protective immunity produced by the procedures used in this experiment were measured by the difference in the antibody level, the packed cell volume, and the parasitemia in the treated animals and the untreated controls. The occurrence of clinical disease in any of the treated animals indicated a lack of sufficient protective immunity.

Because anaplasmosis and babesiosis are enzootic to the outlined ares in Colombia, the cattle were subjected to natural challenge.

All of these tests were prepared to monitor the response

of the various methods of treatment. Serology and laboratory procedures were performed in the Laboratorio de Investigaciones Médicas Veterinarias in Bogotá, Colombia. Total tick collection was performed monthly on 5 animals from each farm and identification was performed in the same laboratory as the serology.

The experiment began in October, 1969 and will continue for approximately 6 months.

The collected data from this experiment will be analyzed by statistical methods when warranted.

Having arrived in Bogotá in September, 1969 to begin a Masters thesis, premunization procedures were began on a group of 4 calves to serve as a pilot test of the effectiveness of simultaneous co-infectious immunity against anaplasmosis and babesiosis with treatment by the 2 new Burroughs-Wellcome drugs, 4A65 and 356-C-61. The four calves were monitored daily for packed cell volume, temperature, complement fixation titers and percentage parasitemia. Meanwhile, several field trips were made to Cali, Girardot and Montería to select 3 cooperating farms which would have a group of approximately 30 three-month old calves. A farm in Buga was selected, but was later eliminated because several of the calves already had positive CF titers for anaplasmosis, therefore, another farm (Hacienda Lucerna) in Bugalagrande was found to be suitable and is presently being used. A farm in Girardot (Hacienda Valencia) was selected and is being

used. Two groups of 30 Zebu and Lucerna calves 2-4 months of age from the 2 above mentioned farms were bled 4 time previous to experimental vaccination and premunization in October, 1969 and all experimental animals are being examined weekly to determine vaccination and premunization results.

Another farm in the Montería area was selected to be used in the project, but was later eliminated because a large majority of the calves had complement fixation titers against anaplasmosis and babesiosis. Due to the high incidence of infection in young calves in the Montería area, a decision was made to purchase 30 Holstein calves in the Savanna of Bogotá and use them on the experiment station at Turipaná as previously described as used on the other farms. The calves were purchased in December, 1969 and were vaccinated and premunized in January, 1970 prior to air shipment to Montería sometime during the second week of February, 1970.

The weekly work schedule has been established as the following:

Monday: Travel to Bugalagrande to collect blood and tick samples from the Hacienda Lucerna calves.

Tuesday: Travel to Girardot to collect blood and tick samples from the Hacienda Valencia calves.

Wednesday: Travel to Montería to collect blood and tick samples from the Turipaná calves.

Friday: Perform packed cell volumes, percentage parasitemias and complement fixation tests and record the results in the L. I. M. V. records in Bogotá.

3. The Relation of Chemotherapy of Anaplasmosis by B-W 356-C-61 with Co-infectious Immunity

The above mentioned experiment is described in the babesiosis section as both anaplasmosis and babesiosis were treated together simultaneously.

B. Anaplasmosis Projected Projects

1. To investigate the possibility that the tick Anocentor nitens transmits bovine anaplasmosis, since it has been found in a large number of incidences on Colombian cattle.
2. To study the pathogenesis of a double infection with Anaplasma marginale and Trypanosoma vivax in intact calves, utilizing serological, clinical pathological, histopathological and immuno-fluorescent methods of study.
3. To look into future possibilities of using an irradiated A. marginale vaccine in cattle.
4. If the immuno-fluorescent study in progress should reveal a particular site of the proliferation of A. marginale to study the ultrastructure of the site in the hopes of finding further clues relevant to the propagation of the organisms.
5. To study the pathogenesis of a triple simultaneous infection with A. marginale, Babesia bigemina and Trypanosoma vivax utilizing serological, clinical pathological, histopathological and immuno-fluorescent techaniques.

6. In vitro culture of Anaplasma marginale in tick intestine tissue culture.

III. BABESIOSIS

A. Babesiosis Projects in Progress

1. A Study of the Pathogenesis of Babesiosis in Intact Calves: Including Serological, Clinical Pathological, Histopathological and Immuno-fluorescent Techniques of Study.

Adequate investigation of the host-parasite interaction has not been thoroughly studied especially as related to the site of propagation of Babesia bigemina within the intact calf.

Objectives:

1. To study the clinical pathological alterations of babesiosis in comparison with the sequential pathological lesions produced by Babesia bigemina.
2. To investigate the possibility of localization and/or proliferation of Babesia bigemina within host tissues and to compare this with serological reactions.
3. To attempt to correlate the localization of the organisms with the sequence of pathological lesions, clinical pathological alterations and serological reactions.

Materials and Methods:

Fifteen, 4-month old, male, Holstein calves were screened for the presence of hemoprotozoal disease by inoculating pooled blood samples into a splenectomized calf, by the CF test for babesiosis and by the preparation of wet mounts. The calves were examined weekly for 12 weeks prior to inoculation of B. bigemina for packed cell volume, percentage parasitemia and general physical state. One week prior to inoculation of B. bigemina, 3 control samples at 2-day intervals were collected for the determination of the following parameters: packed cell volume, hemoglobin content, bone marrow biopsy, total serum protein concentrations, blood glucose values, body temperature, complement fixation titer, blood smears for the percentage parasitemia, serum glutamic oxaloacetic transaminase concentrations, sorbitol dehydrogenase levels, total leukocytes, differential leukocytes, serum protein electrophoresis, direct, indirect and total bilirubin values.

On day 0, the 15 calves were inoculated with an Australian isolate of B. bigemina, maintained in a carrier calf, at a dosage of 5 ml. subcutaneously. All of the above mentioned parameters were performed on all of the remaining calves every other day except for bone marrow biopsies and body temperatures. Bone marrow biopsies were performed on 3 different calves at 2-day intervals and body temperatures were determined twice daily.

Thereafter, one calf was euthanatized every 2 days for

30 days. Two complete sets of tissues were collected. One set of tissue was collected in buffered 10% formalin and the other set was quickly frozen with solid CO_2 onto wooden blocks with Cryoform and stored at -65°C. All tissues were parafin processed, sectioned at 6 mu. and stained with hematoxylin and eosin. Frozen sections of the tissues were made on a Cryostat microtome and indirectly stained with fluorescein labeled andtibodies for B. bigemina for 30 minutes in a humid 37°C chamber, rinsed 3 times in phosphate buffered saline pH 9 and cover-slipped with a carbonate-bicarbonate glycerol solution at pH 9.

Fluorescent antibody conjugates were prepared against bovine gamma-globulin in the following manner: Six adult, New Zealand rabbits were inoculated with bovine gamma globulin prepared by the precipitation of all serum proteins except gamma and beta globulins with ethodin at 4°C, which were decanted. The ethodin was removed by absorption with activated charcoal and centrifugation. The purified, bovine gamma globulin was then concentrated by lyophilization, reconstituted in buffered physiological saline and injected into the rabbits intramuscularly 4 times at weekly intervals. Two weeks after the final injection, the rabbits were exsanguinated and serum was collected. The rabbit serum containing the anti-bovine gamma-globulin was precipitated as described previously and the purified rabbit antibovine gamma-globulin was concentrated by dialysis against polyvinyl-pyrrolidone to the original volume. The globulin fraction was dialysed against

carbonate-bicarbonate buffer 0.5 M pH 9.0 in physiological saline. The protein content of the solution was determined at 280 mu. in a Beckman spectrophotometer Model D B. Fluoresceing-isothiocynate was added at a rate of 0.05 mg. per each mg. of protein to the surface of the globulin fraction with continual stirring for 12 hours at 4°C. Excess fluorescein dye was removed by dual passage through 2 G-25 cooled Sephadex columns and the rabbir anti-bovine gamma-globulin conjugate was collected in 5 ml. fractions and lyophilized and stored at -20°C. The reconstituted conjugate was used to stain the babesiosis specific bovine gamma-globulin which was attached to B. bigemina organisms in the blood smears and frozen tissue sections. The stained specimens were examined with a Leitz Ortholux ultra-violet microscope with a HBO-200 lamp and a BG 12 excitor filter and a 570 barrier filter.

Results:

The results of the clinical, clinical pathological and serological examinations are recorded in the following: Figures 1, 2, 3, 4, 5, 6 and 7.

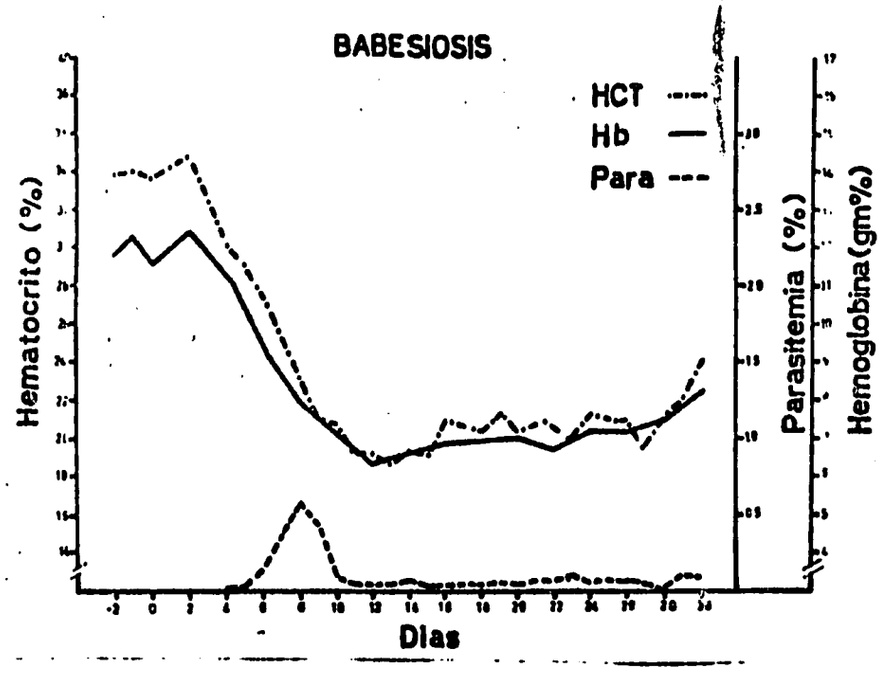

Fig. 1. Packed cell volume, hemoglobin content and percentage parasitemia of peripheral blood are depicted in relation to time.

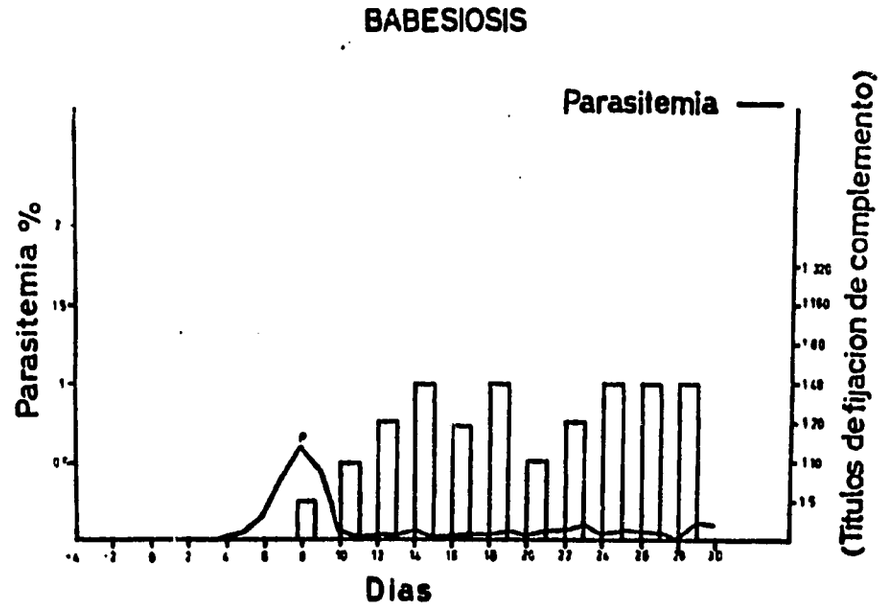

Fig. 2. Percentage parasitemia and complement fixation titers are compared to one another and time.

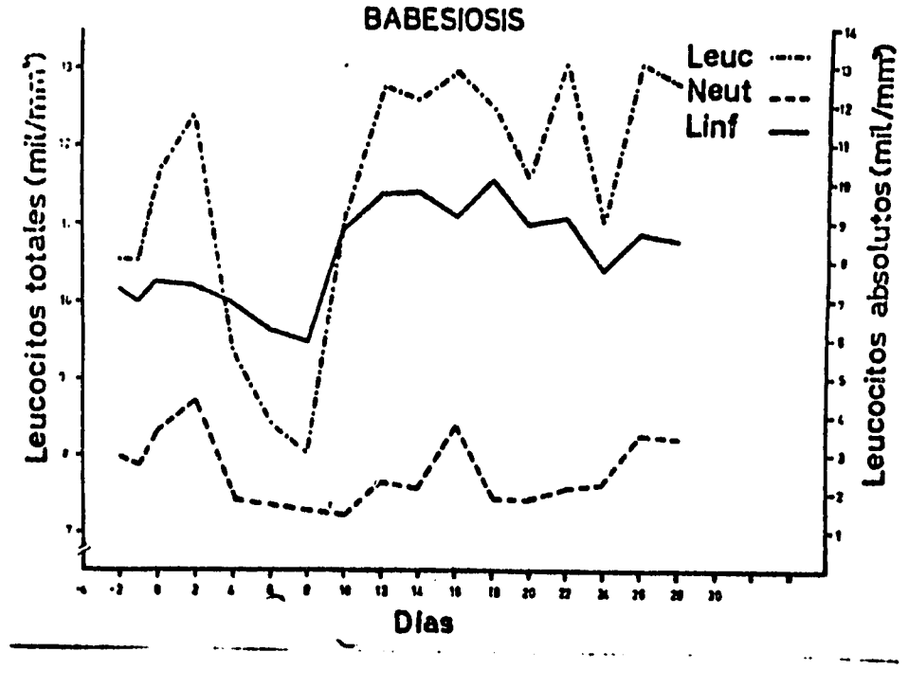

Fig. 3. Total leukocytes, absolute neutrophils and lymphocytes of the peripheral blood are depicted in relation to time.

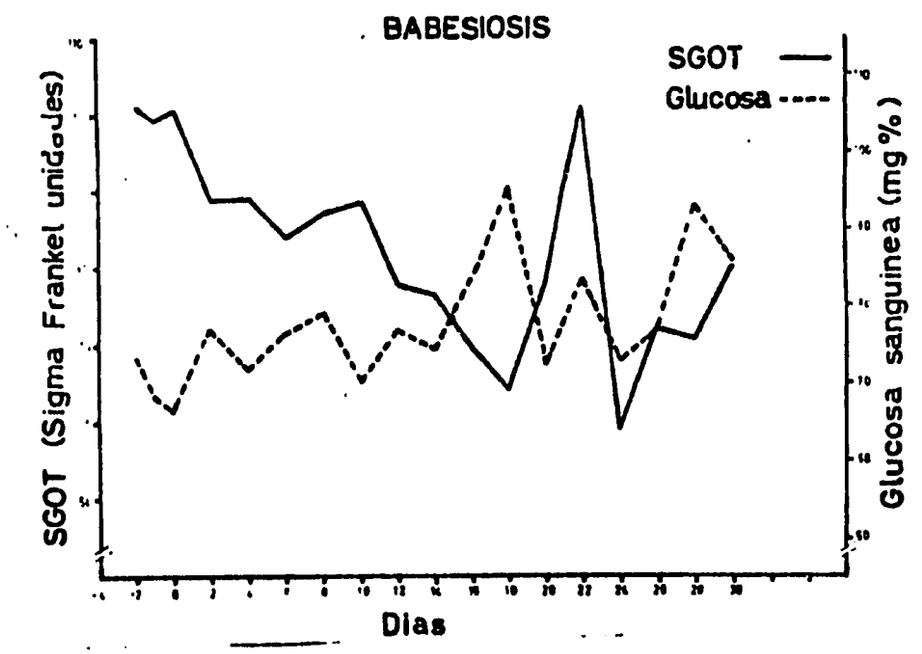

Fig. 4. Blood glucose concentration and serum glutamic oxaloacetic acid transaminase activities are shown in relation to time.

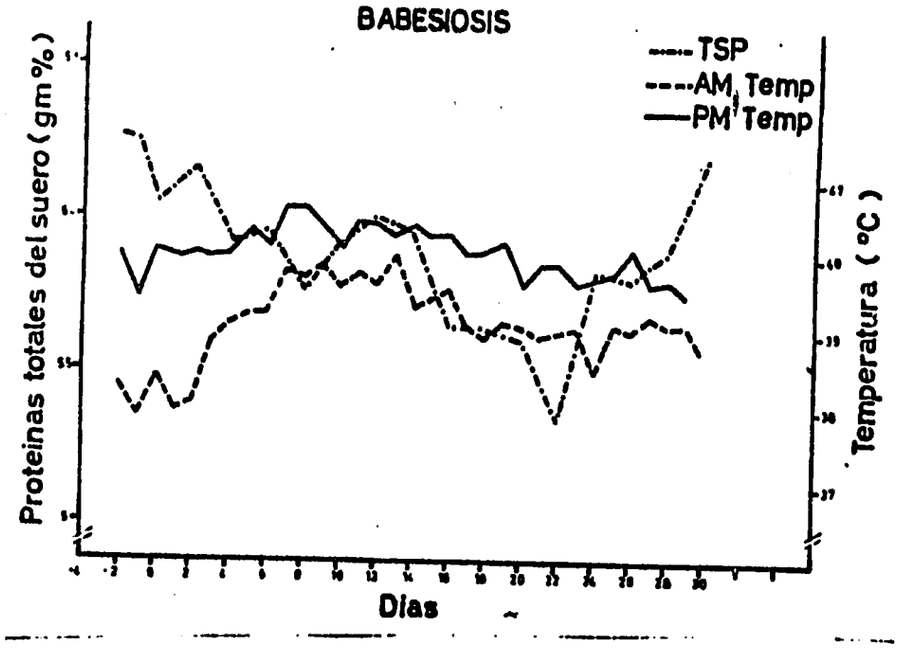

Fig. 5. Body temperature and total serum proteins are demonstrated in relation to time.

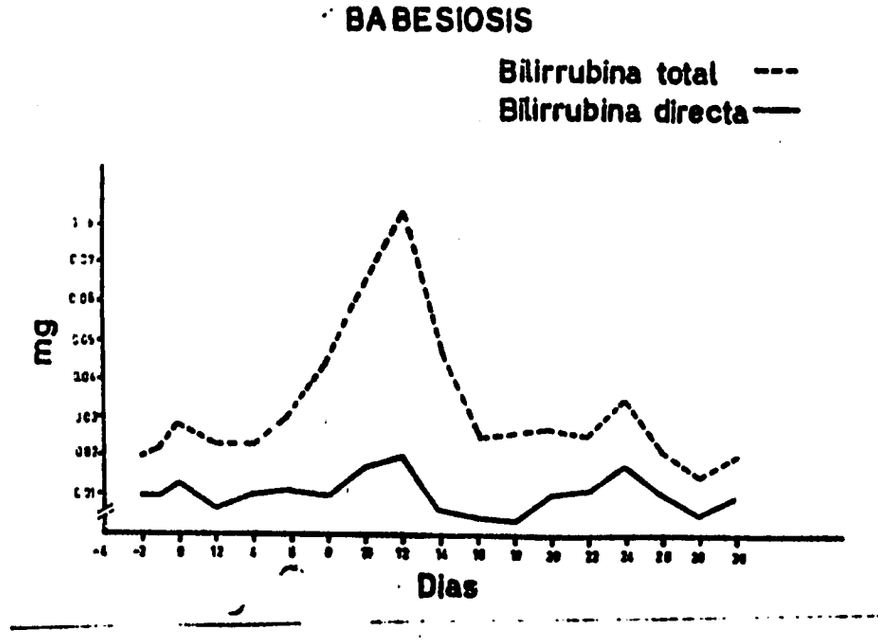

Fig. 6. Total and direct serum bilirubin levels are depicted in relation to time.

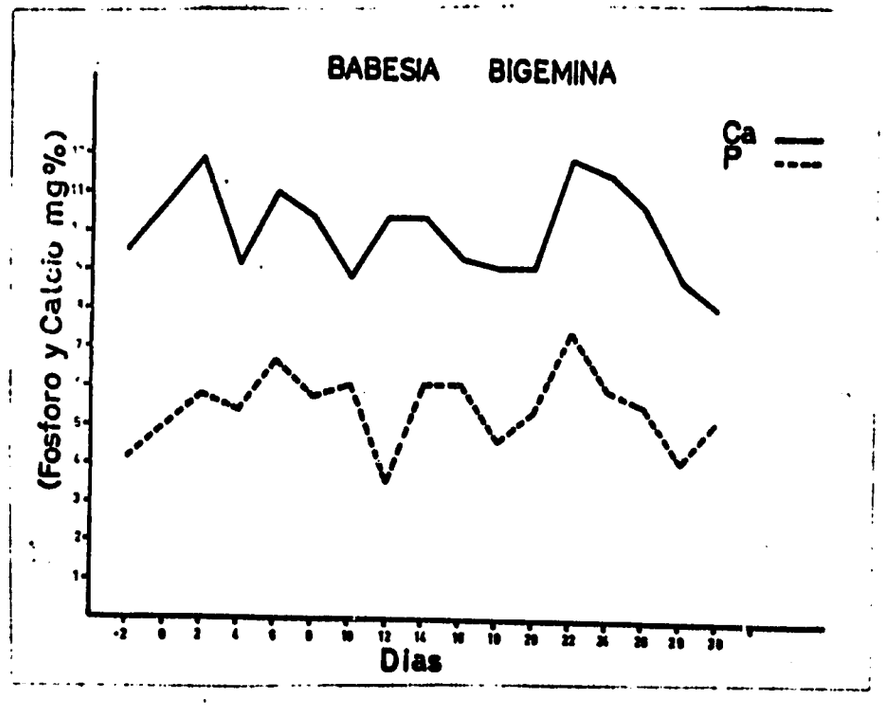

Fig. 7. Serum, clacium and phosphorus levels are shown in relation to time.

The histopathological and immuno-fluorescent studies are not completed, therefore, no analysis can be made at this time.

Discussion:

Since the histopathological and immuno-fluorecent studies are not completed, a total analysis of the experiments is not possible, however, some general conclusions can be made. First, the total leukocytes and absolute number of neutrophils, quickly increased within 2 days after challenge and then suddenly decreased to their lowest respective values on day 8 PI, the day of the highest parasitemia. Perhaps the serum antigen in some way interferes with leukocytes. The hemoglobin and packed cell volume began a rapid decrease on day 2 PI which continued until the packed cell volume reached its lowest value on day 12 PI, which corresponds with the peak of total and direct serum bilirubin concentrations. The peak of parasitemia on day 8 PI also coincides with the first day of complement fixing antibody response which persists in a variable manner until day 30 PI and with the time of the highest body temperature. The host on day 8 PI is showing signs of tissue destruction, but also a favorable response is occurring to eliminate the organism. Blood glucose, serum calcium and phosphorus had variable and inconsistent changes, hence interpretation is difficult. Following day 12 PI the total leukocytes and absolute lymphocytes increase to above pre-inoculation values. The PCV and hemoglobin begin to slowly increase after

day 14 PI. The serum glutamic-oxaloacetic acid transaminase surprisingly decreases throughout the experiment except on days 20 and 22 PI, it was suddenly increased to approximately pre-inoculation values.

2. Chemoprophylaxis Against Bovine Babesiosis

Introduction:

Chemical prophylaxis against bovine babesiosis has not been widely used, although chemotherapy as a prophylactic measure has been used against human malaria (Ryley, 1964). Several drugs have been successfully used in the treatment of acute bovine babesiosis (Joyner et. al., 1964). The mechanism of the action of the majority of chemotherapeutic drugs against babesiosis has not been studied sufficiently, but they undoubtedly exert multiple influences on the Babesia parasites as well as the infected host through various tissue mechanisms and systems which are not presently understood.

Chemotherapeutic drugs apparently act directly on the Babesi parasites causing a decrease in the number, size, vacuolization and morphology of the nucleus and cytoplasm (Nuttall and Hadwer, 1909). The changes in the Babesia parasites apparently occur due to the breakdown of the metabolic and enzymatic processes which ordinarily ends in the death of the organism. Simultaneously, the drug causes changes in the tissue reactivity, stimulates the active mesenchyme

and reticulo-endothelial system, increases the phagocytosis or the number of macrophages, helps to produce defensive substances and increases the immunological properties of the host.

"Babesiacidation" of cattle will be the basic chemotherapeutic prophylactic measure used. It consists of the administration of a babesiacide with prolonged residual action into cattle in curative dosages shortly before exposure (Ryley, 1964). An effective babesiacidal drug will alter the pace of pathological processes, prevent the development of the acute disease and bring about asymptomatic or mild sickness, while not preventing the development of co-infectious natural immunity (Kemron et. al., 1964). If administered at the moment when the parasites begin to multiply in tick-infested cattle or before the first signs of disease begin to appear, the drug would completely stop the development of parasites (Pipano, 1964). The administration of the babesiacidal drug before carrier tick infest the cattle is contraindicated, because the drugs would have a shortened period of activity against the organisms. The search for a new drug which can be used for the administration into susceptible cattle as a prophylactic measure in shorter or longer terms before exposure is obviously needed. The introduction of the drug as a prophylaxis of babesiosis will have a great practical application in the countries where babesiosis is prevalent.

The possibility of chemoprophylaxis against Babesia species infections was investigated by Ryley (1964) who tested the

ability of sparingly soluble salts of drugs with known therapeutic activity to form a drug depot when injected into rats subcutaneously. The drugs which formed a depot, were tested for their ability to prevent clinical babesiosis in splenectomized calves when the animals were later challenged with Babesia divergens. Encouraging results were obtained with quinuronium 5,5' methylene-bis-salicylate and quinuronium embonate.

Pipano (1964) reported that Berenil (GANASEG) given in doses of 5 mg/kg during the incubation period of infection with Babesia berbera in calves had the effect of lengthening the incubation period and decreasing the severity of the disease. Treatment of clinically affected calves produced a decrease in the temperature within 24 to 48 hours, but the parasites persisted in the blood up to 4 days after treatment, indicating that the drug caused inhibition of the multiplication of the parasites rather than their destruction. One or 2 doses did not interfere with the co-infectious immunity of recovered calves. The administration of the drug 4 days prior to infection did not confer any protection.

Barnett (1965) studied the chemotherapy of Babesia bigemina in a series of experiments in splenectomized cattle in which the effects of treatment, subsequent relapses and immunity were noted. Amicalbalide in doses of 10 mg/kg was very effective, but the treated animals subsequently proved susceptible to reinfection. Co-infectious immunity followed the administration of 1.5 mg/kg or less, but some fatal relapses occurred. Homidium bromide was

active at 1 mg/kg or more, but reduction of the number of parasites after treatment was slow and one severe relapse occurred after treatment. Plenamidine isothionate (13.3 mg/kg) acted rapidly and co-infectious immunity followed treatment, and clinical relapse did not occur ever with doses at 8-10 mg/kg. Acaprin (1 mg/kg) acted rapidly, producing co-infectioys immunity with clinical relapse, but with a dosage of 0.75 mg/kg a fatal relapse occurred after treatment. Berenil in doses of 3.5 mg/kg or less caused a a rapid elimination of the parasites which were not demonstrable thereafter. The animals were immune for up to 109 days after treatment. Berenil (5 mg/kg) produced a sterile cure and the animal was completely susceptible to reinfection 68 days later.

The objectives in the experiment are to evaluate the prophylactic action of the 4A65 babesiacidal drug by experimental and tick-borne challenge of treated animals, and to determine the duration of the prophylactic period in the treated animals.

Materials and Methods:

The Burroughs-Wellcome experimental compound 4A65 (3,3¹ Bis-2-imidazolin-2yl-carbanilide dihydrochloride)* was used in the experiments. The 4A65 drug was received from the Burroughs-Wellcome company as water soluble powder and was dissolved in sterile distilled water as 10% solution and was used within 2

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* Burroughs-Wellcome and Company, Tuckahoe, New York.

days after preparation. The experiments were undertaken at the Instituto Colombiano Agropecuario laboratories located at Laboratorio de Investigaciones Médicas Veterinarias (Bogotá), Tibaitatá, the experimental stations in Palmira and Buga (Valle del Cauca).

Eight experiments were conducted to evaluate the prophylactic and therapecutic effects, dosage, route of injection, toxicity and the response of the animals injected with the 4A65 drug. A total of 70 animals were divided in several experimental groups at different altitudes and places and the following experimental designs were used to ascertain the above stated objectives.

EXPERIMENT 1.- Three 85 Kg., male, Holstein calves, 4 months old at Bogotá (alt. 2700 m.), Colombia, were used in the preliminary trial for the evaluation of the 4A65 drug. Two calves were given 3 mg/kg of 4A65 intravenously and the other calf served as control. Observations for drug toxicity were made for 30 minutes following injection. The 2 treated calves and the non-treated control calf were challenged 21 days after injection of the drug with 100 ml. containing 1.2 Gm Na EDTA/liter of blood containing 0.1% Babesia bigemina intravenously. All calves were examined weekly for 3 months before challenge and daily after challenge for the following parameters: packed cell volume, percentage parasitemia, temperature and complement fixing antibody titer. Body weights were made weekly.

EXPERIMENT 2.- Four 65 Kg., male Holstein calves, 3 months old at Bogotá (alt. 2700 m.), Colombia, were used in the evaluation

of the 4A65 drug. Three calves were given 2 mg/kg of 4A65 intramuscularly and the remaining calf served as control. Observations for drug toxicity were made for 30 minutes following injection. The 3 treated calves and the non-treated control calf were challenged 21 days after injection of the drug with 100 ml. containing 1.2 Gm. of Na EDTA/liter of B. bigemina and B. argentina carrier blood intravenously. All calves were examined weekly for 3 months before and twice daily after challenge for packed cell volume, temperature and parasitemia. Complement fixing antibody titers were done weekly before and daily after challenge.

EXPERIMENT 3.- The experiment was executed as described in Experiment 2 except that the four calves were challenged 45 days after injection of the 4A65 drug.

EXPERIMENT 4.- Six 35 Kg., male, Holstein calves, 5 months old at Palmira (alt. 1000 m.), Colombia, were used for a field evaluation of 4A65. Two calves were given 3 mg/kg and 4 calves were given 2 mg/kg of 4A65 intravenously. Two calves served as controls. Observations were made for drug toxicity for 120 minutes. All experimental calves were maintained in tick-free units for 25 days after which they were field challenged with Boophilus microplus ticks naturally infected with Babesia spp. All calves were examined weekly before and twice weekly for 231 days after tick-borne challenge for packed cell volumes, temperature, parasitemia and complement fixing antibody titers. Tick collection and identification was performed monthly.

EXPERIMENT 5.- Thirteen 138 Kg. (72-304 range) male, Ayrshire, Brown

Swiss and Holstein calves 7½ months-old (2-10 months range) at Bogotá (alt. 2700 m.), Colombia, were used in a field co-infectious immunity evaluation at 4A65 drug. The calves were given 5 ml. each of citrated blood from a carrier calf containing Anaplasma marginale, B. bigemina and B. argentina subcutaneously. All calves were treated 5 days after inoculation with 4A65 for babesiosis at a dosage of 1 mg/kg intramuscularly and Burroughs-Wellcome drug* 356-C-61 for anaplasmosis at a dosage of 5 mg/kg intravenously. Observations were made for drug toxicity for 30 minutes following injection. The calves were maintained on tick-free premises until field challenge with Boophilus microplus ticks naturally infected with A. marginale, B. bigemina and B. argentina 93 days later in Puerto Asis (alt. 260 m.), Putumayo, Colombia. The calves were examined weekly for 2 weeks before challenge and weekly thereafter for packed cell volumes, parasitemia, body weights and complement fixing antibody titers against anaplasmosis and babesiosis.

EXPERIMENT 6.- Fifteen 125 Kg. female, Holstein calves, 3 to 6 months old at Buga (alt. 1000 m.), Colombia were used to study the prophylactic effects of 4A65 against babesiosis and 356-C-61 against anaplasmosis. The calves were simultaneously given 4A65 at a dosage of 2 mg/kg intramuscularly and 356-C-61 at a dosage of 5 mg/kg intravenously. Observations for drug toxicity were made for 30-60 minutes following injection. Two weeks following treatment, the 15 calves were exposed to Boophilus microplus ticks

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* Burroughs-Wellcome Co., Tuckahoe, N.Y., B-W Drug No. 356-C-61

naturally infected with A. marginale, B. bigemina and B. argentina. The calves were examined weekly for 2 weeks before and weekly after field challenge for 195 days for packed cell volumes, parasitemia, body weight and complement fixing antibody titers against anaplasmosis and babesiosis.

EXPERIMENT 7.- Twelve 100 Kg., male Holstein calves, 4-6 months old at Palmira (alt. 1000 m.), Colombia, were used in a field co - infectious immunity against babesiosis and anaplasmosis. Two calves served as non-treated controls. The experimental calves were maintained in tick-free units and were subjected to fecal examination for helmenthiasis and treated 18 days before blood-borne exposure with Thiabendazole* to eliminate intestinal parasites. Fecal examinations were performed monthly throughout the experiment. Ten calves were given 5 ml. each of blood (Na EDTA 1.2 gm/liter) from a carrier calf containing A. marginale, B. bigemina and B. argentina. Eight days following exposure, the calves were given 4A65 at a dosage of 2 mg/kg intramuscularly and observations for drug toxicity were made for 30-60 minutes following the injection of the drug. Sixteen days following artificial blood-borne challenge, the calves were give 356-C-61 drug at a dosage of 4 mg/kg and 16 days later, the treatment with 356-C-61 was repeated at a dosage of 5 mg/kg intravenously. Four weeks after the artificial blood-borne challenge, the 12

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* Merck and Company

calves were placed on premises infested with B. microplus naturally infected with A. marginale, B. bigemina and B. argentina. Weekly examinations were made for packed cell volumes, parasitemia, body weight, temperature and complement fixing antibody titers against babesiosis and anaplasmosis.

EXPERIMENT 8.- The experiment was carried out to determine the therapeutic efficacy of the 4A65 drug against acute bovine babesiosis caused by different species and isolated of Babesia parasites. Seven male, 83 Kg., Holstein , 3-6 months old calves were used at Bogotá (alt. 2700 m.), Colombia. All calves were experimentally infected with Babesia spp. When signs of acute babesiosis were evidenced by anemia, parasitemia, hemoglobinuria and temperature, the animals were treated. The 4A65 drug was injected as a 10% aqueous solution in one animal at a dosage of 1 mg/kg subcutaneously, in 2 animals 1 and 2 mg/kg respectively intravenously and in 4 animals 1 and 2 mg/kg intramuscularly. Two to 60 minutes after drug treatment, any signs of toxicity were recorded. The percentage of recoveries was determined by the return to normal parameters measured, e.g. packed cell volume, temperature, body weight, complement fixing antibody titers and physical state as before the acute infection.

Results and Conclusions:

At the present time, some experiments in this report are still in progress. Some preliminary results (Experiment 1 and 3) are

recorded in Figure 1 and 2. However, on the basis of the data of the experiments terminated, some conclusions can be drawn. The new experimental drug 4A65 has prophylactic activity, but has also shown some toxicity. Animals injected with 4A65 up to 46 days before challenge are highly resistant to blood-borne challenge with Babesia spp. Non-injected control animals died when exposed to this same challenge. It was found that toxic symptoms were most pronounced when 4A65 was injected intravenously in the dosage of 3 mg/kg. Two animals of 5 injected died after injection with 3 mg/kg with the following signs of reaction: excessive salivation, lacrimation, difficulty in breathing,urination and defecation. These signs were observed one minute following injection and fatal results occurred in from 2 to 10 minutes.

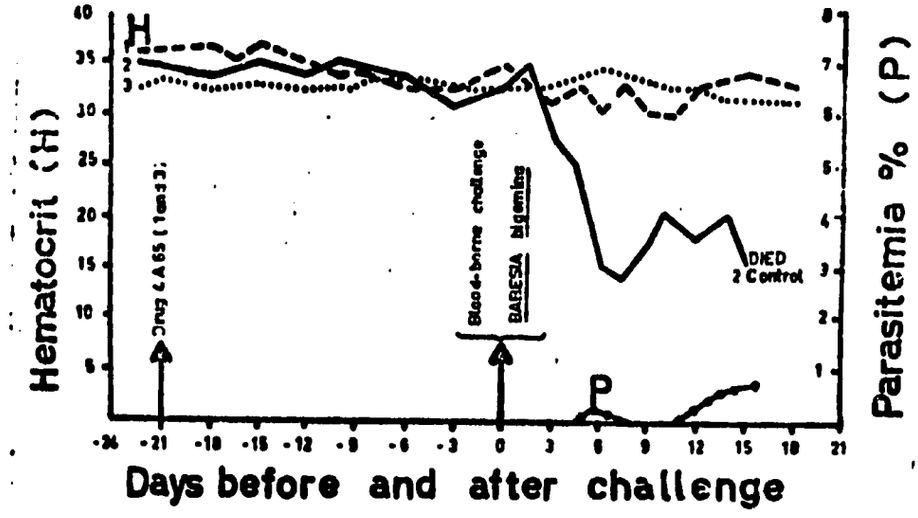

Fig. 1. Graphic representation of percent of parasitemia (P), hematocrit values (H) and their relationships to the prophylactic treatment with the new experimental drug No. 4A65 (21 days) and blood-borne challenge with Babesia bigemina (Experiment 1).

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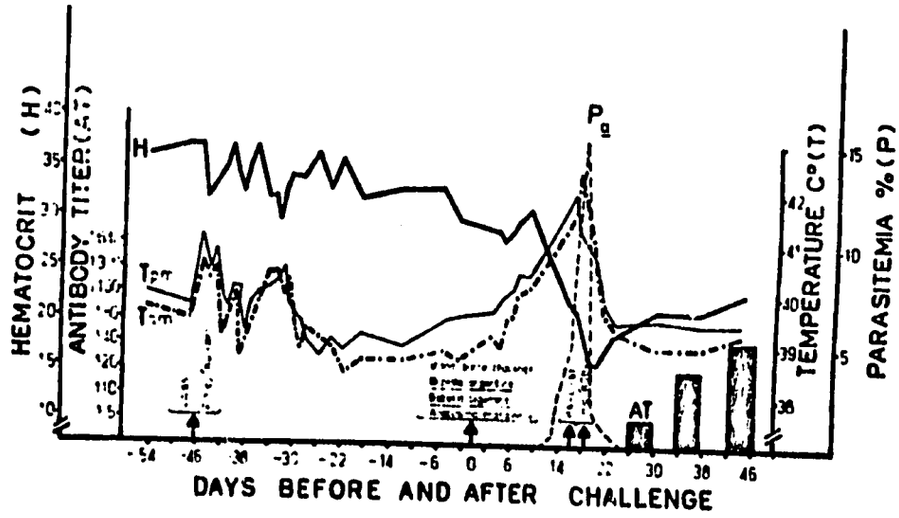


Fig. 2. Graphic representation of percent parasitemia (P), hematocrit values (H), body temperature (T) and antibody titers (AT) and their relationships to the prophylactic treatment with the new experimental drug No. 4A65 (46 days) and blood-borne challenge with Babesia bigemina, Babesia argentina and Anaplasma marginale (Experiment 3).

Tam = Temperature A.M.

Tpm = Temperature P.M.

When the dosage was reduced to 1-2 mg/kg and the route of injection changed from I.V. to I.M., the reactions to 4A65 were much less severe. Since all of the experiments are not completed, the conclusions cannot be made as to the specific dosage to be used and to the duration of protection at this time. The experiments now in progress should answer these questions when they are terminated. Animals injected with 4A65 prophylactically, show resistance to babesiosis, but not to anaplasmosis. It is concluded that 4A65 is specific for bovine babesiosis, but has no effect on anaplasmosis.

3. Co-Infectious and Sterile Immunity Against Bovine Babesiosis

Introduction:

Attempts to produce immunity in cattle against Babesia infections have been undertaken by several investigators with varying success. Although a sterile immunity was described in cattle which had recovered from an infection from Babesia argentina (Riek, 1964), attempts to produce artificial immunity with killed-Babesia parasites have resulted in only partial protection (Mahoney, 1967). The classical concept of premunition or co-infectious immunity proposed by Sergent et. al. (1924) was derived from these workers' observations on Babesia bigemina infection that the immunity persists as long as the animal remains a latent carrier of the Babesia infection. If parasites disappeared as a result of auto-

sterilization, effective chemotherapy, the animals become fully susceptible.

A disease comparable in some aspects with malaria is bovine babesiosis, so the experience obtained in developing a resistance against Plasmodium parasites could be exploited in developing a resistance against Babesia infections since the mechanism of immunity in both blood infections are very similar. The present situation in regard to the mechanism of immunity in hemoprotozoal diseases is as follows: A sterile immunity was described in rats which had recovered from an infection with Plasmodium berghei (Corradetti, 1950), however, attempts to produce a sterile immunity with killed-Plasmodium parasites have resulted in only partial protection. Following immunization of rats with crude Plasmodium berghei antigens, Zuckerman et. al. (1965) obtained a moderate degree of resistance, whereas Corradetti et. al. (1966) did not.

Plasmodium berghei-infected mice cured by drug therapy become resistant to a challenge infection (Box and Gingrich, 1958). The inconclusive results obtained with killed vaccine have been attributed to the loss or denaturation of a soluble antigenic fraction during the preparation of the vaccines. Furthermore, the parasites may have been too rapidly destroyed in the host to elicit a discernible protective effect. Sinton (1939) postulated that the amount and the rate of development of immunity to malaria may depend upon the amount and duration of antigenic stimulation. Experiments were designed to elucidate more on the phenomenon of

sterile and co-infectious immunity to Babesia so that causes of ontoward results of premunization and vaccination against Babesia may be elucidated and better methods of producing vaccine devised.

Objectives:

To determine the infectivity and virulence of blood from animals either recivered from or reacting to Babesia spp. and used for production of live-Babesia vaccines. To study the response of vaccinated animals under the Colombian conditions of tropical environment. To evaluate the duration and degree of co-infectious immunity by tick-borne challenge. To determine the degree and duration of sterile immunity in vaccinated animals with killed-Babesia vaccines. To compare the results of sterile immunity and degree of protection with the results of the co-infectious immunity trials.

Materials and Methods:

A total of 60 Holstein-Friesian male calves with an average age of 150±20 days were used in this experiment. Calves selected at random were divided into 4 groups, according to the experimental design used to evaluate the immuno-serological responses. The first group (A) consisted of 10 male, 85 Kg., Holstein, 3-month old calves wh ch were premunized with Babesia bigemina, Babesia argentina and 10 weeks later were exposed to tick-borne challenge (Boophilus microplus naturally infected with Babesia bigemina and Babesia argentina). The second group (B), consisting of 10 male,

90 Kg., Holstein-Friesian, 4-month old calves, were not premunized, this group was used as a control. The third group (C) of 10 splenectomized calves with an average age of 160±20 were inoculated with the same amount of blood as the calves from the first and second group. The fourth group (D) consisted of 20 male, 95 Kg., Holstein-Friesian, 4-month old calves subdivided into 4 groups A, B, C, D. Four intact calves in each group were vaccinated with respective vaccines and one splenectomized calf was not vaccinated and used as control. The experiments were carried out at Palmira-ICA experimental station in Valle del Cauca (alt. 1000 m.). The percentage of parasitemia (P), packed cell volume (PCV), antibody titer (AT), temperature (T), mortality (M) were used as the basis of comparing the reaction produced after vaccination and tick-borne challenge.

Results and Discussion:

The results of the experiment designed to study the mechanism of co-infectious and sterile immunity are recorded in figures 3, 4, 5, 6, 7, 8, 9 and 10.

Animals premunized with blood collected from patent carriers of Babesia bigemina and Babesia argentina develop slight post-inoculation reactions which were manifested by parasitemia and anemia. After 3 weeks, these animals recovered from anemia and parasitemia was not detectable. When the animals were exposed to tick-borne challenge, Boophilus microplus naturally infec

with Babesia bigemina and Babesia argentina, they were highly resistant to babesiosis, but all of these were infected with Anaplasma marginale. Ticks collected from these animals were identified as Boophilus microplus and Dermacentor nitens.

A control group of non-premunized intact animals was exposed to tick-borne challenge at the same time as the principal premunized group. These control animals became infected with babesiosis and anaplasmosis after 4-10 weeks of exposure and 25% of them died. Further loses were prevented by treatment against anaplasmosis and babesiosis. The conclusion can be made that the ticks present carriers of both Babesia spp. and Anaplasma marginale and that the effective control of tick-borne diseases at this location cannot be made unilateral.

A second control group of splenectomized animals used to test the infectivity of the blood used in the premunization experiment developed acute babesiosis from 2-10 days after blood-borne challenge. As a result, 90% of these animals died. Further loses were prevented by 2 additional treatments against babesiosis. The data of this experiment shows that the blood used for premunization was highly infective. Additional experimentation is needed to determine the minimum amount of blood sufficient to produce infection without severe post-premunization reactions. In addition, more experimentation is also needed to provide more information on strain pathogenicity of Babesia parasites used in premunization studies. Experiments designed to answer these questions are now in progress.

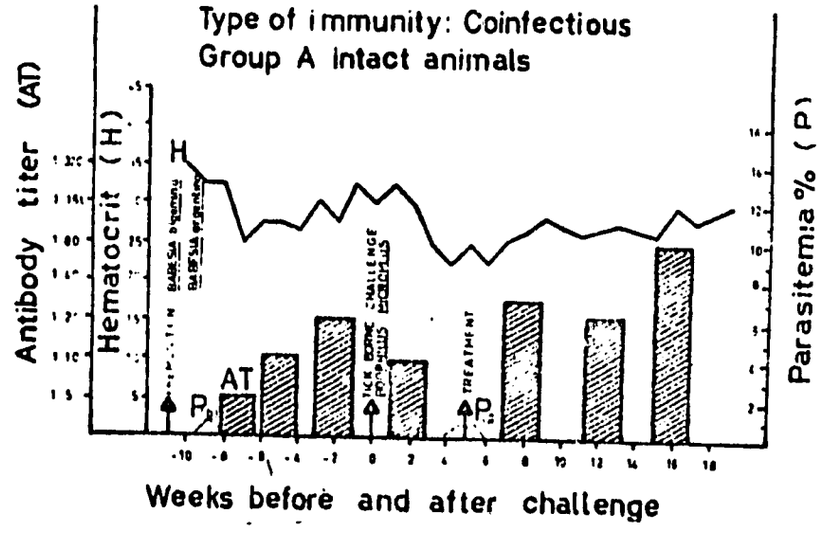


Fig. 3. Graphic representation of mean values of percent of parasitemia (P), hematocrit values (H), antibody titer (AT) and their relationships to the premunization with Babesia bigemina and Babesia argentina and tick-borne challenge (Boophilus microplus naturally infected with Babesia bigemina and Babesia argentina).

P(b) = Parasitemia with Babesia bigemina and Babesia argentina

P(a) = Parasitemia with Anaplasma marginale

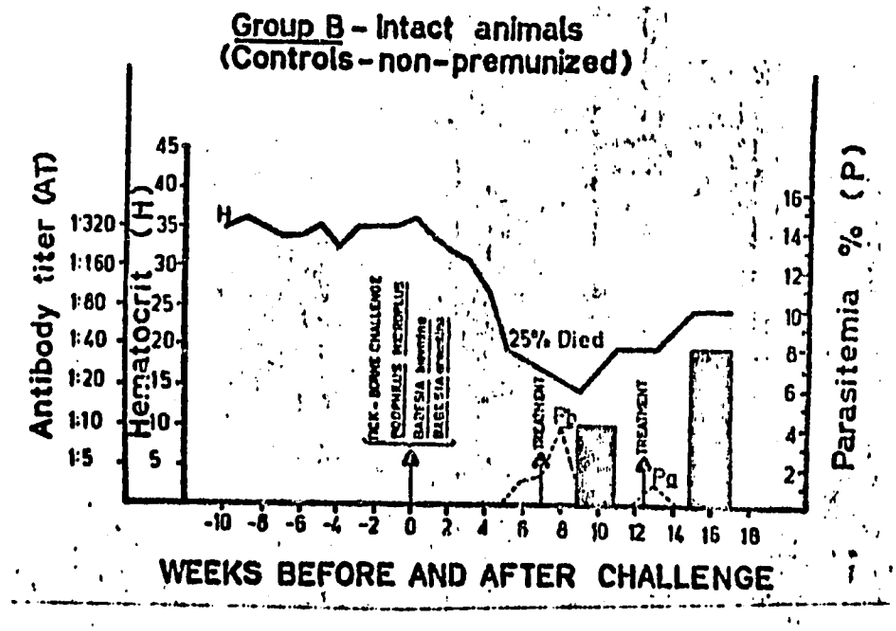


Fig. 4. Graphic representation of the mean values of percent of parasitemia (P), hematocrit values (H) and antibody titer (AT) and their relationships to the tick-borne challenge (Boophilus microplus naturally infected with Babesia bigemina and Babesia argentina).

P(b) = Parasitemia with Babesia bigemina and Babesia argentina.

P(a) = Parasitemia with Anaplasma marginale.

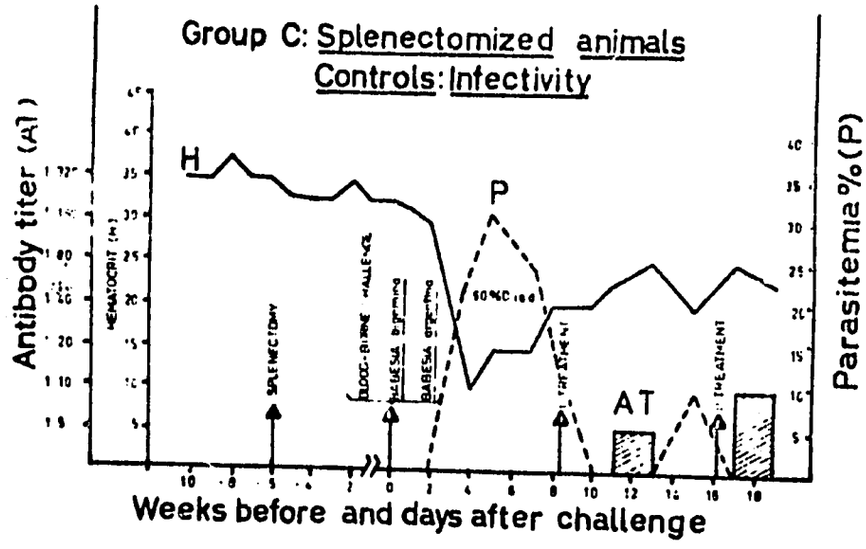


Fig. 5. Graphic representation of the mean values of the percent parasitemia (P), hematocrit values (H) and antibody titer (AT) and their relationship to splenectomy and blood-borne challenge with Babesia bigemina and Babesia argentina.

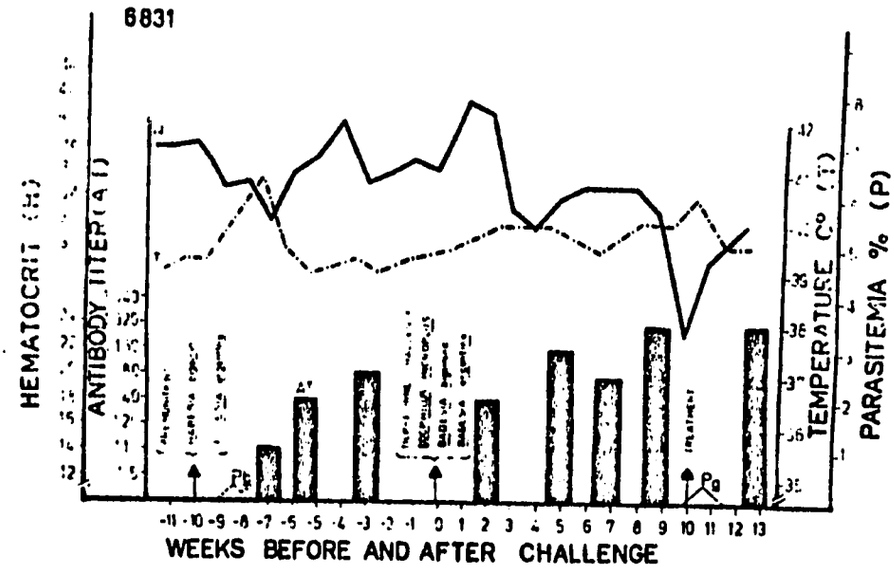


Fig. 6. Graphic representation of percent of parasitemia (P), hematocrit values (H), temperature (T) and antibody titer (AT) and their relationships to premunition with Babesia bigemina, and Babesia argentina and 10 weeks later with tick-borne challenge (Boophilus microplus naturally infected with Babesia bigemina and Babesia argentina)

P(b) = Parasitemia with Babesia bigemina and Babesia argentina

P(a) = Parasitemia with Anaplasma marginale

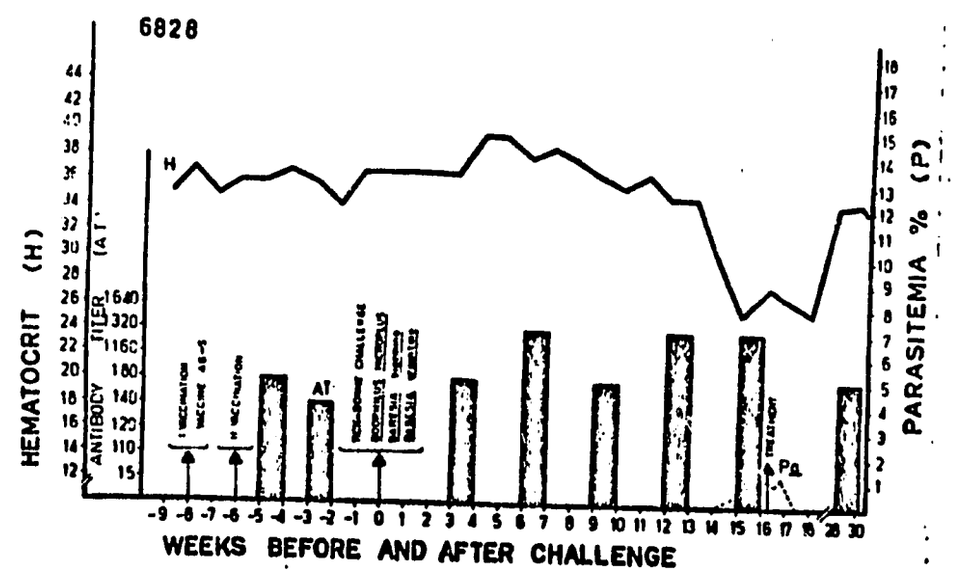


Fig. 7. Graphic representation of the percent of parasitemia (P), hematocrit values (H) and antibody titer (AT) and their relationships to vaccination with vaccine AG-S and eight weeks later tick-borne challenge (Boophilus microplus naturally infected with Babesia bigemina and Babesia argentina).

P(a) = Parasitemia with Anaplasma marginale.

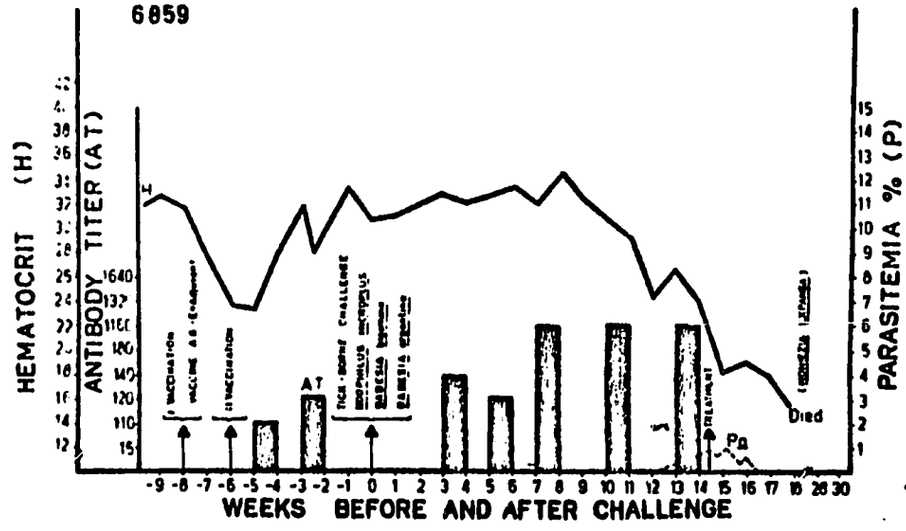


Fig. 8. Graphic representation of the percent parasitemia (P), hematocrit values (H) and antibody titers (AT) and their relationships to the vaccination with AG-E plus adjuvant and 8 weeks later tick-borne challenge (Boophilus microplus naturally infected with Babesia bigemina and Babesia argentina).

P(a) = Parasitemia with Anaplasma marginale.

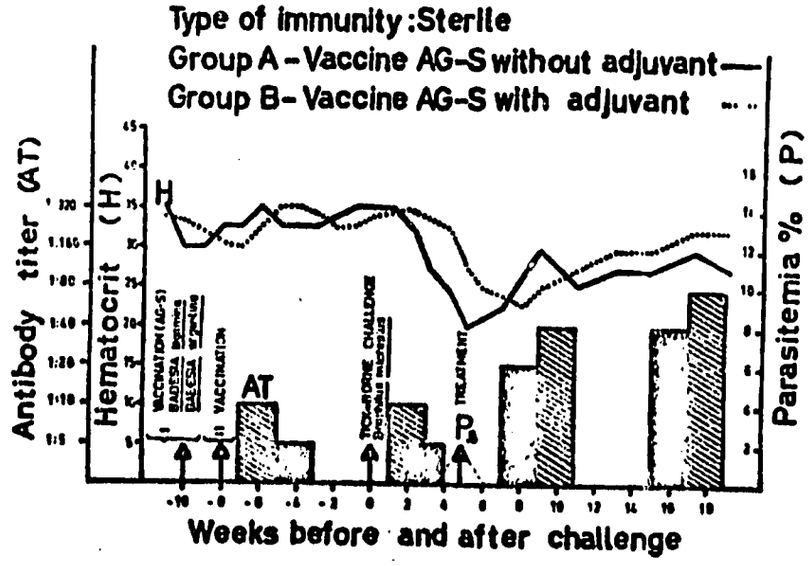


Fig. 9. Graphic representation of the mean values of the percent of parasitemia (P), hematocrit values (H) and antibody titer (AT) and their relationships to the vaccination with vaccine AG-S and 10 weeks later with tick-borne challenge (Boophilus microplus naturally infected with Babesia bigemina and Babesia argentina).

P(a) = Parasitemia with Anaplasma marginale.

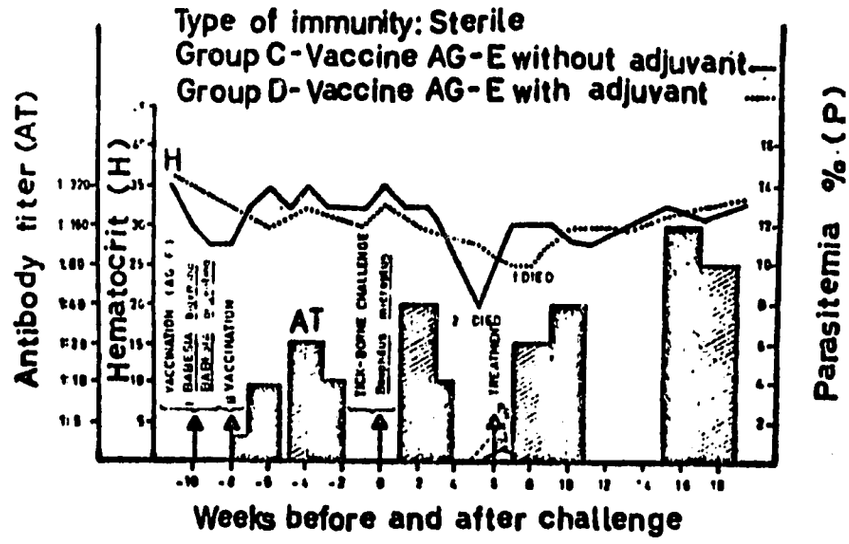


Fig. 10 Graphic representation of the mean values of the percent of parasitemia (P), hematocrit values (H) and antibody titer (AT) and their relationships to the vaccination with vaccine AG-E and 10 weeks later with tick-borne challenge (Boophilus microplus naturally infected with Babesia bigemina and Babesia argentina).

P(a) = Parasitemia with Anaplasma marginale

P(b) = Parasitemia with Babesia bigemina and Babesia argentina

Results of the experiments designed to ascertain the mechanism of sterile immunity demonstrate the possibility of producing this type of immunity by killed-Babesia vaccine. Because some animals in this experiment died from anaplasmosis and helminthic parasites results are inconclusive and specific conclusions cannot be made at this time. Part of this experiment is currently being repeated. However, results of the experiment with AG-S vaccine plus adjuvant demonstrated a high degree of protection against tick-borne challenge. No losses occurred in the animals tested. However, non-vaccinated control animals died.

On the basis of observations made with these experiments, final conclusions can be drawn that some degree of sterile immunity exists in Babesia infections. To understand the exact mechanism of this type of immunity, more work needs to be done. Degree of resistance and duration of immunity in relationships to different environmental conditions, strains, pathogenecity of Babesia organisms, and quality and quantity of tick-borne challenge needs to be determined. Susceptibility and resistance of the host also plays an important part in the tropical areas and needs further study.

4. Ultrastructure of Babesia bigemina

Resumen:

Es bien conocida la importancia que en los paises tropicales

tienen las enfermedades producidas por los hematozoarios y las pérdidas económicas que de ellas se derivan.

Este ensayo forma parte del estudio de identificación de diferentes cepas de babesias encontradas en Colombia y sus características morfológicas. Se trata de revelar aparte de los aspectos morfológicos ya conocidos por la Babesia en microscopia de luz, lo que nos revela en el microscopio electrónico. La cepa que se revela en el microscopio electrónico fué obtenida pura en animales a los cuales se les encontró alta parasitemia de B. bigemina en una región del Valle del Cauca. Después la cepa fue inoculada en terneros esplenectomizados en el Laboratorio de Investigaciones Médicas Veterinarias (LIMV) de Bogotá. Cuando la parasitemia fue alta (25%) la sangre fue recolectada en heparina y el material se envió a Palmira donde se fijó en glutaraldehido y se guardó en un preservativo antes de iniciar su procesamiento en M/E.

El material en estas condiciones se llevó a la Universidad del Valle, donde se inició el proceso de fijación, inclusión, corte y preparación en el Laboratorio de Microscopia Electrónica de la Universidad del Valle.

La ultraestructura revelada por el microscopio electrónico de la B. bigemina está compuesta por una membrana plasmática, muy delicada y de difícil preservación lo que hace que no sea revelada en forma muy clara. Su núcleo, sin embargo, se conserva bien con doble membrana nuclear y se encuentra casi siempre colocada en la periferia del citoplasma y no revela nucleolos.

Una de las partes revela una estructura en forma de cono de la cual derivan pequeños canales que van a terminar a las vacuolas de sostenimiento del parásito según los denomina Rudsinska y que según Hepler y colaboradores corresponden a cuerpos polares que intervienen en el movimiento del parásito dentro del eritrocito.

El reticulo ergoplástico se revela muy bien, pero no se encuentran en el citoplasma, mitocondrias y pigmentos. Estudios mas completos para tratar de conocer mejor su ultraestructura, se encuentran en progreso.

Introduction:

Bovine babesiosis is an infectious and transmissible disease manifested by progressive anemia associated with the presence of intra-erythrocytic protozoan parasites which belong to the genus Babesia. Their systematic position and their relation to other blood protozoa vary with the authority and is still a subject of disagreement among taxonomists. However, babesiosis has been recognized as a specific and severe infectious entity in many areas of the world. Babesiosis is particularly prevalent in the subtropical environments, where there are ticks. In the countries such as the United States, babesiosis was wiped out during the tick-vector (Boophilus annulatus) eradication program.

Babesiosis is still of great importance as a treat to the livestock industry in Colombia, South America. At the present time, the prevalence of babesiosis in Colombia is difficult to

determine. According to the published reports, the disease exists as a mixed infection of Babesia bigemina, Babesia argentina and Babesia major (Velásquez, 1938).

The fundamental nature of B. bigemina, B. argentina and B. major has not been adequately determined. Critical and extensive light microscope observations have revealed that the organism assumes many forms, varying from budding structures to the usually pear-shaped body (Riek, 1964). Babesia bigemina is a large parasite, 4-5 mu. in length by about 2 mu. in width, round forms, 2-3 mu. in diameter. The organisms are characteristically pear-shaped and lie in pairs forming an acute angle in the red blood corpuscle. Babesia argentina is a small organism, the pyriform forms are 2-1.5 mu. in diameter and usually lie in the center of the erythrocyte. T' .orms most frequently seen are vacuolated rings with the nuclear material at one point on the periphery of the circular zone at the cytoplasm. The paired pyriform parasites usually form an obtuse angle in the erythrocyte. Babesia major resembles Babesia bigemina, but is smaller, measuring about 2.6 by 1.5 mu. The paired pyriform parasites are somewhat elongated, meet at an acute angle and tend to be located toward the middle of the corpuscle. Spherical forms, usually less than 2 mu. in diameter, are similar to those seen in B. argentina infection.

With the advent of electron microscopy, many of the differences of this species can be more elucidated and the morphologic characteristics more clearly defined. The reproduction of Babesia is

not clear and the mechanism of feeding and other factors of host-parasite relationships are not quite clearly understood, therefore, more recently, many attempts have been made to study the ultrastructure of Babesia canis (Bayer and Denning, 1961), Babesia rodhaini (Rudzinska and Trager, 1962), Babesia caballi and Babesia canis (Simpson et. al., 1962), Babesia caballi (Ristic, 1966) have been studied. No reports could be found in the literature of an electron microscopic study of Babesia bigemina, Babesia argentina or Babesia major. Therefore, an attempt was made to study the ultrastructure of these bovine species of Babesia.

An electron microscopic study of several species of malarial parasites disclosed that these intracellular organisms feed on their host cells by phagotrophy or pinocytosis, engulfing parts of the erythrocyte cytoplasm into food vacuoles (Rudzinska et. al., 1960; Rudzinska and Trager, 1957; Rudzinska, 1959). To find out whether other intracellular parasites, particularly those which live in erythrocytes, might feed in the same way bovine Babesia parasites were examined by means of electron microscope. Although the exact mechanism of feeding of intracellular parasites is not clear and the interpretation is controversial. Hepler et. al. (1966) do not agree with the explanation of Rudzinska and Trager (1959) on the feeding mechanism of malaria. However, numerous papers on the fine structure of malaria parasites in the intra-erythrocytic stage (Aikawa, 1966; Cox and Vickerman, 1966;

Duncan et. al., 1959; Fletcher and Maegraith, 1962; Jerusalem and Heiner, 1965; Ladda et. al., 1965; Trager, 1966 and Trager et. al., 1967) have confirmed the feeding mechanism earlier reported by Rudzinska and Trager (1959). In general, it was believed that the uptake of nutrients from the host cell by intracellular parasites is generally supposed to occur across the cell membrane of the parasites. In some instances, it has been necessary to assume that rather large molecules can pass through the cell membrane. In malaria parasites, for example, it seemed as if intact hemoglobin must permeate the plasma membrane of the parasite from the host erythrocyte for hemozoin, the insoluble residue of the digestion of hemoglobin, occurs within the body of the parasite. Electron microscopic studies have revealed, however, that in two species of malaria parasites, Plasmodium lophurae (Rudzinska and Trager, 1957) and Plasmodium berghei (Rudzinska and Trager, 1959) hemoglobin enters the parasites not across the plasma membranebut through its invagination to form pinocytotic food vacuoles. Digestion of the content of the food vacuoles follows two patterns (Rudzinska et. al., 1965). In Plasmodium falciparum as in Plasmodium lophurae, digestion takes place within vacuoles in which hemozoin, the residue of hemoglobin accumulates. In Plasmodium vivax, P. ovale, P. gonderi and P. coatneyi, vesicles are pinched off from the food vacuoles and digestion takes place in small vesicles.

In our study, we have given special attention to the ultra-

structure of Babesia bigemina erythrocytic stage and we have attempted to access the significance and function of their specialized organelles and structures. The following objectives are considered: (1) Morphology of parasites (ultrastructure), (2) Reproduction and (3) Feeding mechanisms.

Material and Methods:

Babesia bigemina fue obtenida de animales infectados naturalmente en una región del Valle del Cauca y fue purificada en terneros esplenectomizados hasta que la parasitemia fue alta (25%). La sangre fue recolectada inmediatamente con anticoagulante (EDTA) y preservada bajo refrigeración.

La sangre heparinizada fue fijada con glutaraldehido (solución buffer pH 7.2, 0.1 molar) centrifugando por 3 veces, una vez cada hora a 1.000 r.p.m. y 0°C. Se lavó con una solución buffer-fosfato sucrosa, centrifugando 4 veces durante una hora. Después se colocó en solución durante 30 minutos a 0°C. Se hicieron secciones de 1 mm. de espesor y el material se fijó con osmio-buffer fosfato pH 7.2, 0.1 molar durante 5 horas y se lavó con una solución buffer-sucrosa pH 7.2, 0.1 molar.

El proceso de deshidratación se hizo con alcohol de 75% por otros 15 minutos y finalmente alcohol de 95% por otros 15 minutos. Luego se hicieron 3 cambios de 15 minutos cada uno en alcohol absoluto y otros 3 cambios de 15 minutos en óxido-propileno, dejando el material en solución de óxido-propileno y maraglass a partes iguales durante dos horas y maraglass solamente por 12 horas.

La inclusión se hizo en maraglass a una temperatura de 60°C durante 48 horas, utilizando cápsulas BEEN LADD. El material incluído fue cortado con un ultramicrotomo PORTER BLUMM MT 2 a 500 armstrongs, utilizando cuchillas de diamante.

La coloración se hizo con acetato de uranillo al 2% durante 15 minutos, lavando con agua bidestilada y colorante de Millonig (solución de tartrato) durante 30 minutos y lavados en agua bidestilada. El espécimen se colocó en rejillas de 100 huecos, protegido por una película de carbón. Las fotografías se tomaron en un microscopio JOEL tipo JEM-7A.

NOTA.- También se utilizó la coloración de WATSON (citrato de plomo y citrato de sodio) originalmente, pero el contraste obtenido no fue muy bueno.

Resultados:

Frotis Teñidos de Sangre: La Babesia bigemina en frotis teñidos de sangre con la coloración de Giemsa-Wright aparece como un parásito intracelular colocado en el centro del hematie, revela una forma piriforme y aparecen generalmente en forma de parejas unidas en ángulo agudo por sus extremos afilados, aunque también pueden tener formas esféricas. Su componente citoplásmico se tiñe de azul y el núcleo toma una coloración rojiza.

M. oscopio Electrónico:

1. Tamaño: Son grandes, en ocasiones ocupan todo el glóbulo rojo fácilmente detectables en microscopios de luz y M/E, llegan

a medir longitudinalmente de 6-3 micras y transversalmente de 2-4 micras.

2. Membrana Plasmática: Densa, no definida pero revela ser simple. Entre esta y el reticulum endoplásmico se ve un espacio claro de unos 2500 armstrong, lo cual no define si la primera corresponde al eritrocito y la otra sea propia del parásito lo que explica posiblemente un proceso de parasitación por invaginación.

3. Reticulum Endoplásmico: Tiene aspecto vesicular compuesto de gránulos que se unen formando un entramado entre la membrana citoplásmica y nuclear. Los gránulos son de diferente tamaño y llegan a tener mayor densidad que los componentes del eritrocito. Las partículas mas densas que se observan dan origen a pensar que son mas ricas en ribonucleoproteina que corresponden según Rudzinska a cuerpos de Palade.

4. Cuerpos Polares: Son organelos de 0,3-0,5 micras de diámetro que se encuentran como componentes del citoplasma y que se diferencian del resto por su mayor densidad y estructura definida. Generalmente aparecen en número de 2-3 y se comunican directamente con la membrana citoplásmica por medio de canales a la porción cono del parásito. Rudzinska dice que estos canales interviene en el proceso de alimentación del parásito, siendo los cuerpos polares vacuolas de alimentación o sostenimiento del parásito. Hepler et. al. piensan que los cuerpos polares interviene en el movimiento del parásito dentro del eritrocito.

5. Vacuolas: Se han encontrado en poco número dentro del parásito. Aparecen como formaciones claras, esféricas u ovoides y morfología irregular.

6. Núcleo: Se ha visto en casi todos los parásitos, ocupando 1/3 - 1/4 del volumen de este. La membrana nuclear es simple pero densa, de un espesor de 800-1000 asmstrong, suelen alcanzar hasta 2 micras de diámetro. Está colocado generalmente en el centro del parásito con una densidad similar a la de la estructura del huésped. No revela nucleolos teniendo una estructura granular y homogénea.

Reproducción: Se han sugerido por diversos autores, dos formas de reproducción de las Babesias. Rudzinska en Babesia rodhaini, sugiere un tipo de reproducción ppr gemación, pero también encontró formas que sugieren un tipo de reproducción por división binaria.

Para Babesia bigemina, nosotros pensamos que la forma de reproducción por gemación es mas común. En estos casos, el parásito es capaz de formar largos pseudópodos que parten del citoplasma estableciéndose unión entre este y el parásito a través de lo cual pasan componentes del citoplasma y del núcleo que contribuyen a la formación del nuevo parásito. En cortes se han visto estas formas evolutivas con una composición muy similar a la del parásito La forma reproductiva por división binaria también ha sido observada y en este caso correspondería a la misma forma de división celular por mitosis en la que los componentes celulares se van situando a uno y otro lado hasta la formación del nuevo parásito, aunque esto no ha sido revelado en forma muy clara.

Discussion:

The fine structure of Babesia bigemina resembles closely that of Babesia rodhaini (Rudzinska and Trager, 1962), Babesia caballi (Simpson et. al., 1966). In both Babesia caballi and Babesia canis as well as Babesia bigemina, typical mitochondria are lacking. According to Rudzinska and Trager (1962), mitochondria is also lacking in Plasmodium berghei; however, in both a structure is is present, composed of concentric membranes similar to a certain type of mitochondria formed in some stages of spermatogenesis in Helix pomatia (Beams and Tahmisian, 1954). It is assumed, therefore, that the concentric organelle might perform mitochodrial functions (Rudzinska, 1959). The absence of mitochondria was also reported in Babesia canis (Bayer and Denning, 1962). Babesia possesses as does Plasmodium berghei an elongated double-membraned vacuole with enlarged bulb-like ends. The function of this vacuole is unknown. It is assumed that it might represent a reservoir of fluids which could be of importance for an intracellular parasite surrounded by a dense cytoplasm of the erythrocyte (Rudzinska, 1959).

According to Rudzinska and Trager (1962) in Plasmodium berghei, the two double-membraned structure differs distinctly. The double-membraned vacuole has only one pair of membranes, whereas the concentric structure is always composed of two or more pairs. In Babesia bigemina, the latter structure often possesses only one pair of membranes and is less frequently two or more pairs. The two structures are sometimes difficult to distinguish in Babesia

bigemina and the number of transitional forms suggest that both might represent stages in the development of just one structure.

The endoplasmic reticulum is in the form of vesicles bound in most cases by a smooth and only occacionally by a rough surfaced membrane representing a rather primitive type of this organelle (Porter, 1957). This applies actually to all other organelles in Babesia bigemina. They are less differentiated and less complicated than in Plasmodium species.

According to Rudzinska and Trager (1962), the primitive organelles are also the way of reproduction by budding and binary fission in Babesia. Both forms of reproduction were reported previously in a somewhat different way by investigators using light microscopy (Nuttall and Graham-Smith, 1908 and Wenyon, 1926). According to their observations, reproduction starts with the formation of two and occassionally of four small buds. The two or four buds grow by incorporating more and more of the nuclear and cytoplasmic matter of the parent until its whole content is exhausted, The results in the formation of two or four equal daughter cells. Thus, reproduction of Babesia would start with budding and end up with binary fission. Budding would represent only a preparatory step for binary fission. It would be very difficult to know whether similar processes are involved in reproduction of Babesia species, since the observations described here are based on fixed and thin section material (Rudzinska and Trager, 1962).

The body of Babesia bigemina as well as Babesia rodhaini is covered by a single plasma membrane. In this respect, Babesia differs from malarial parasites which are surrounded by two membranes (Rudzinska et. al., 1960, and Rudzinska and Trager,1957). A double plasma membrane is characteristic also for malarial merozoites (Rudzinska, 1961). The membranes are newly formed during reproduction and laid around each merozoite. They remain throughout the whole life span of the parasites. In Babesia bigemina, the buds and the daughter cells posses a single plasma membrane deriving from the existing plasma membrane surrounding the parent organism.

It is of interest that Babesia rodhaini (Rudzinska and Trager, 1962), an intracellular parasite of red blood cells of the mouse, feeds on the host cell in the same way as malarial parasite. It engulfs droplete of host erythrocyte cytoplasma through invaginations of the plasma membrane with the subsequent formation of food vacuoles. In Babesia, however, no pigment hemozoin was found in the food vacuole nor outside it. Apparently, the pathway of digestion of engulfed host cytiplasma is different in this parasite. It might be that the utilization of ingested hemoglobin is more complete in Babesia than in Plasmodium parasites.

In spite of the difference in the mechanism of food digestion, the manner of feeding on the host cell is stressed before the same (Rudzinska et. al., 1965). In all malarial parasites and in Babesia it is done by ingestion of droplets of the surrounding host cyto-

plasm into food vacuoles through invaginations of the parasite's plasma membrane. In this way, the whole host cytoplasm becomes gradually incorporated into the body of the parasite, where is is digested.

The supposition that Babesia bigemina, an intraerythrocytic parasite, might feed on its host cell in the same way as Babesia rodhaini as reported by Rudzinska and Trager (1962) did not find support in the present electron microscope study. As in malarial parasites, large oval bodies of the same density and structure as the host cytoplasm were not found in Babesia. There is no doubt that they represent parts of the erythrocyte engulfed into the body of the malaria parasites through invagination of the plasma membrane leading to the formation of food vacuoles. Both parasites are confined exclusively at certain stages in their life cycle to the same type of host cells to erythrocytes.

This specific requirement in two not very closely related organisms (Malaria and Babesia) resulting in similar feeding suggests that the erythrocyte or some substances present in its cytoplasm might be responsible for the way of feeding of both organisms. There is good evidence to support such a supposition (Rudzinska and trager, 1962).

The ingestion of droplets of the surrounding medium seems to be a widespread phenomenon recently shown by means of electron microscopy in a great variety of cells (Bennet, 1956, Dempsey, 1953, Palade, 1956, Pease, 1956, Rhodin, 1954 and Yamada, 1955).

Lewis (1931) first introduced for it the term pinocytosis, stressed its possible significance. Of great importance is the finding that pinocytosis can be induced by certain substances (Brandt, 1958, Champan-Anderson and Holter, 1955, Edwards, 1925, Mast and Doyle, 1933 and Schumaker, 1958).

The first observation on engulfment of fluid in amoebae were made by Edwards (1925). He also found that this process can be induced by certain inorganic salts. More detailed information on pinocytosis was performed by Lewis (1931) on macrophages and cells in tissue cultures and thereafter by Mast and Doyle (1933) on several species of Amoeba. The latter investigators established that pinocytosis can be induced not only by inorganic salts, but also by calcium gluconate and by egg albumin.

Experimental studies on pinocytosis undertaken by Halter (1957) and Halter and Marshall (1954), Marshall et. al., (1959) showed that a number of proteins are very effective as inducers. From the studies on the mechanism of pinocytosis, it seems that inducers act directly on the plasma membrane by adhering and being bound to it (Brandt, 1958 and Schumaker, 1958). It is assumed that a subsequent change in the physical state of the plasma membrane results in its invagination. Droplets of the surrounding medium are trapped into the invaginations which become finally cut off as vacuoles.

All the findings strongly suggest that very similar processes are taking place in feeding of malarial and Babesia parasites (Rudzinska and Trager, 1962). It is reasonable to assume to

assume that some proteins and salts in the erythrocyte cytoplasm which represents the surrounding medium of both parasites act as inducers of pinocytosis and as such, are responsible for the invagination in the plasmarmembrane and engulfment of droplets of the surrounding host cytoplasm. Since the whole process takes place inside the host cell, we might regard it as intracellular pinocytosis. The way of taking in droplets of host cytoplasm by intracellular pinocytosis is the simplest and probably the most efficient way of using the host's body as food supply.

The utilization of the content of the food vacuoles seems to be more extensive in Babesia than in malarial parasites (Rudzinska and Trager, 1962). In all plasmodia residue of hemoglobin digestion in the form of hemozoin can be detected whereas in Babesia the digestion of hemoglobin appears to be nearly complete since no traces of hemozoin could be found in electron micrographs.

In contrast to a previous study of Babesia canis reported by Bayer and Denning (1961), Simpson et. al. (1963) reported that Babesia canis like other protozoan, was confined by a definite cytoplasmic membrane. Additional observations made by the same authors include a detailed description of the endoplastic reticulum and the presence of intracytoplasmic organelles having a double membrane. In regard to these structures, Babesia canis, Babesia caballi and Babesia rodhaini appeared to be similar to Babesia bigemina. A simple confinding plasma membrane, however, has been described in the latter organism, whereas Babesia caballi

and Babesia canis posses a double membrane. The ultrastructure of Babesia canis and Babesia caballi were shown to be identical in the work reported earlier.

Neither mitochondria nor Golgi apparatus has been identified in Babesia. However, it is suggested with Babesia rodhaini, intracytoplasmic organelles with double membrane may possibly assume activities. The presence of cristae-like strands in some of these structures suggests that they are pporly differentiated mitochondria. It is believed that large numbers of cristae are only to be expected in tissue cells a high energy turnover.

The conoid, as first described and named by Gustafson et. al. (1954) in Toxoplasma and later by Hepler et. al. (1966) in Plasmodium fallax, appears on the anterior end of the parasite and is generally thought to be concerned with the attachment and subsequent penetration of the parasite into the host cell (erythrocyte). We find this an attractive hypothesis, though a final proof of such an activity remains incomplete.

The paired organelles observed in Babesia bigemina which appear similar to the paired bodies of malarial parasites and to the toxonomes of Toxoplasma and the sarconemes of sarcocysts have also been implicated in the process of penetration of the parasite into host cell.

Garnahm et. al. (1960) have suggested that the paired organelles of observed in sporozoites of malaria might contain proteolytic enzymes which would assist in lysing the plasmalemma of the host

cells and thus allow the parasite to enter. Again, this theory has not been proven but seems possible.

Our own work shows that the paired organelles and neighboring dense bodies are among the first organelles to disappear in newly penetrated parasites. Their early appearance implies that the organelles are used in the initial stage of infection, and possibly in the actual entry process itself. Their close structural association with the conoid and the presence of channerls to the outside through the region of the conoid, further suggest that the paired organelles are active in penetration or feeding mechanisms.

While we continually observe irregularities in the surface of Babesia bigemina, we have never discovered processes which could be interpreted as host cytoplasm engulfment or phagotrophy as described by Rudzinska and Trager (1957 and 1959) and Rudzinska et. al. (1965) in growing erythrocytic forms. Nor have we been able to identify pinocytosis at the surface of the limiting membrane of the Babesia bigemina parasits.

In addition, we have not observed within the parasite, vesicles containing what we could identify as host cell cytoplasm. Our findings suggest that the erythrocytic stage feeds by a process involving diffusion of necessary metabolites and precursors in the form of small molecules from the host cytoplasmic reticulum as described by Hepler et. al. for malaria parasites. The presence of endoplasmic reticulum of small vesicles irregularly

shaped and often contain a matrix of higher density than the ground substance of the cytoplasm and ribosomes indicates that it is capable of synthesizing its own ATP and its own structural proteins and enzymes required for metabolic reaction. The typical mitochondria are missing and it might be assumed that the concentric structure performs mitochondrial functions (Rudzinska and Trager, 1962). Neither within the vacuoles nor outside it in the cytoplasm, could the pigment hemozoin be detected suggesting that Babesia bigemina is able to digest hemoglobin completely than can malarial parasites.

5. Diagnóstico de Babesiosis Bovina en Frotis Delgados de la Sangre

Resumen:

La dificultad para el diagnóstico de la babesiosis bovina que casi siempre se presentó en baja parasitemia ha motivado numerosas investigaciones relacionadas con su diagnóstico. Nosotros continuando esa inquietud por la búsqueda de una técnica mejor, presentamos "Diagnóstico de Babesiosis Bovina en Frotis Delgados de la Sangre," con el ánimo de que sea útil a los Centros de Diagnóstico del país.

Nuestro trabajo se realizó en el Laboratorio de Investigaciones Médicas Veterinarias del Instituto Colombiano Agropecuario en Bogotá. Utilizamos 32 animales de raza Holstein, machos la mayoría y algunas hembras cuyas edades fluctuaron entre los 5 y 9 meses. De estos, un número de 13 se esplenectomizaron. Todos los ani-

males clinicamente sanos fueron chequeados previamente, antes de entrar en experimentación, para los datos normales de hematocritos y la corroboración negativa o positiva a parásitos.

Los animales e inocularon posteriormente con Babesia argentina, Babesia bigemina y Babesia major. Inmediatamente después de la inoculación, el trabajo de rutina consistió en la colección de sangre para los frotis de sangre y para pruebas serológicas acompañadas de la temperatura rectal. La sangre se colectó de la vena yugular en tubos estériles con anticoagulante (oxalato de amonio potásico, citrato de sodio, disodium ethylenediamine tetraacetate) para los frotis y en tubos sin anticoagulante para las pruebas serológicas. Cuando comenzó el incremento de la temperatura rectal, se tomaron también muestras de sangre de la oreja.

Los frotis se hicieron inmediatamente después de tomar la muestra de sangre y luego a intervalo de 2 horas con la sangre al medio ambiente. Con la sangre refrigerada se efectuaron frotis cada 24 horas durante una semana.

Para la diagnosis se usaron métodos de enriquecimiento a partir de a sangre con citrato de sodio con el cual se obtiene una mayor concentración de los parásitos en la capa superior de sobrenadante en las primeras horas de sedimentación. Una modificación de este método se usó utilizando centrífuga.

Los resultados interesantes fueron obtenidos y una nueva técnica fue desarrollada que ayudara el diagnóstico de babesiosis

aguda y el trabajo futuro para el mejoramiento de esta técnica de frotis delgados de la sangre. El significado de este trabajo será discutido.

Introducción:

La babesiosis bovina e infecciones de algunos otros protozoarios son diagnosticadas por demstración de los parásitos en frotis de sangre, coloreados con Giemsa (Shute, 1955, Saal, 1964) o colorantes similares (MacNeal, 1922, Kingsley, 1935, Roe et. al., 1940, Conn, 1953 y Baker, 1958). Mahoney y Saal (1961) introdujeron la técnica de la gota gruesa que tiene la ventaja de una concentración alta de organismos, pero una coloración satisfactoria frecuentemente es difícil, muchos de los parásitos se deforman y se dificulta identificarlos entre los restos celulares a menudo presentes. Las especies de Babesia también pueden ser coloreadas con colorantes fluorescentes como la Naranja acridina (Winter, 1967) usando la misma técnica que Schiffer (1962) para células hematopoiéticas, que hace visibles todas las células nucleadas pero no los eritrocitos ni trombocitos. Pero son técnicas algo mas dispendiosas y requieren de un microscopio ultravioleta. Seddon (1952) recalcó las dificultades experimentales para hacer una diagnosis de babesiosi por el examen microscópico en frotis delgados de sangre. En babesiosis aparentemente clínica, las infecciones por Babesia bigemina podrían ser reveladas por un examen de rutina en frotis delgados de sangre, pero las producidas por

Babesia argentina muchas veces no son reveladas aun mediante un examen cuidadoso. En nuestro trabajo, estos hallazgos fueron confirmados pronto y se encontró un método más práctico para detectar estos organismos en la sangre de animales infectados. Esto se requería como una base del estudio para determinar, con un grado razonable de seguridad, el porcentaje de infección en animales clínicamente afectados o nó.

Objetivo:

Evaluar un nuevo método de frotis delgado para la diagnosis de infección de babesiosis.

Materiales y Métodos:

El presente trabajo se efectuó en el Laboratorio de Investigaciones Médicas Veterinarias (LIMV) del Instituto Colombiano Agropecuario en Bogotá. Se utilizaron 32 animales de raza Holstein, machos la mayoría y algunas hembras cuyas edades fluctuaron entre los 5 y 9 meses. De estos animales, un número de 13 se esplenectomizaron. Todos los animales clinicamente sanos fueron chequeados previamente antes de entrar en experimentación para los datos normales de hematocritos y la corroboración negativa o positiva mediante frotis de sangre y pruebas serológicas. Se usaron las pruebas de fijación del complemento, aglutinación del látex, hemoglutinación y precipitación, todas ellas para detectar anticuerpos de Babesia en animales con infecciones agudas y crónicas. El gran problema es el aislamiento y tipo propio del antígeno y técnica

sofisticada para la ejecución de las pruebas serológicas.

Se formaron 5 grupos de acuerdo a la especie de Babesia o de la procedencia de la sangre que se emplearía para la inoculación. El primero y segundo grupos, ambos de 12 animales, se infectaron con cepas puras de Australia de Babesia bigemina respectivamente. Al tercer grupo de 4 animales, se le aplicó sangre de animales bovinos portadores de Montería. Al grupo cuarto, de 2 animales, sangre de bovino de la región de Fusagasugá al quingrupo, también de 2 animales, sangre de bovino del Valle del Cauca.

Inmediatamente después de la inoculación, el trabajo de rutina consistió en la colección de sangre para los frotis de sangre y pruebas serologicas. Otro dato diario fue la temperatura rectal. La sangre se colectó de la vena yugular en tubos estériles con anticoagulante (oxalato de amonio potásico, citrato de sodio, disodium ethylenediamine tetraacetate) para los frotis y en tubos sin anticoagulante para las pruebas serológicas. Cuando comenzó el incremento de temperatura rectal, se tomaron muestras de la vena yugualr y de la oreja en las horas de la mañana y de la tarde.

Los frotis se hicieron inmediatamente después de la toma de la muestra de sangre y luego a intervalo de 2 horas con la sangre al medio ambiente. Con sangre refrigerada se efectuaron frotis cada 24 horas durante una semana.

Para la diagnosis del parásito, se usaron métodos de enriquecimiento a partir de la sangre con citrato de sodio. El fundamento de estos consistió en agregar a un cilindro con 25 ml. de una

solución de citrato de sodio al 1% y una igual cantidad de sangre venosa, la cual se agitó y se dejó verticalmente en reposo para permitir la sedimentación de los elementos de la sangre. Después de la sedimentación se succionó cuidadosamente con pipeta Pasteur la capa de plasma citratada del sobrenadante y se prepararon frotis que se colorearon con Giemsa.

Se hizo una modificación para acelerar la sedimentación de los elementos de la sangre utilizando centrífuga. En este caso, 3-4 ml. de una solución de citrato de sodio al 2% se mezclaron en tubos de centrífuga con la misma cantidad de sangre. El contenido de cada tubo se agitó y centrifugó 3-5 minutos a 500-700 r.p.m. El sobrenadante se pipeteó hasta donde comenzaba la capa de sedimento, se transfirió a un segundo tubo y nuevamente se centrifugó por 15-20 minutos a 1500-2000 r.p.m. El sobrenadante claro se eliminó y del sedimento se prepararon láminas que se colorearon con Giemsa.

También se prepararon láminas después de unas 3 horas de reposo de la sangre a partir del fondo del tubo, de la mitad del contenido y de la superficie superior del mismo.

Los frotis se efectuaron sobre láminas con una pequeña gota de sangre en un extremo que se extendió cuidadosamente con otra mediante ligero movimiento oblícuo. Se secaron inicialmente al aire y luego se fijaron con alcohol metílico absoluto 100%. En nuestro trabajo usamos el colorante Giemsa stain (Gradwohl Laboratories, St. Louis, Missouri) el cual se usó con soluciones a diferentes pH (5.1, 6.7,

6.8, 7.1, 7.2 y 7.3) de aguas destiladas, desmineralizadas, de consumo y de fosfato buffer monobásico, los que se prepararon usando dos gotas de Giemsa stain por cada 1 ml. de las diferentes aguas que se utilizaron para obtener el colorante diluído. Los tiempos de exposición de este colorante sobre el frotis fueron de 10, 15, 20, 25 y 30 minutos.

Resultados:

De los 32 animales, 25 fueron positivos en los frotis; 11 por Babesia bigemina pura de Australia; 6 por Babesia argentina pura también de Australia; 4 por Babesia bigemina y Babesia major con la sangre de Montería y 4 por Babesia bigemina y Babesia argentina con sangre de las regiones de Fusagasugá y del Valle del Cauca. En los grupos tercero, cuarto y quinto, hubo infecciones mixtas de una o dos especies de Babesia con Anaplasma marginale. La evolución de la Babesia bigemina tuvo siempre un período mas mas corto (6-10 días) que la Babesia argentina (15-18 días) y cada vez la evolución se presentó mas pronto mediante los pases de las cepas de un animal a otro. Se observó en las muestras parasitadas que la Babesia bigemina alcanzó siempre una parasitemia mas alta que la Babesia argentina. La forma predominante de Babesia bigemina fué bigeminada en ángulo agudo pero también se observaron formas redondas, ovales, en peras y ameboides de 4-5 micras de longitud por 2 micras de ancho cuando la B. bigemina no es redonda. Cuando es redonda, 3-5 micras. La Babesia

argentina presentó siempre bajas parasitemias con formas redondas pequeñas predominates localizadas en el centro del eritrocito y formas pequeñas en ángulos obtusos de 1.5-2.4 micras de diámetro. Los animales esplenectomizados respondieron en un menor tiempo con un mayor incremento de la parasitemia sobre los animales sanos. En animales con temperatura normal, nunca se vió el parásito.

En frotis directos hechos con sangre de la oreja hubo un mayor porcentaje de parásitos que en frotis hechos a partir de sangre de la yugular. Muchas veces, este porcentaje fue de 1% en frotis con sangre de la yugular y de 1-2% en frotis hechos directamente con sangre de la oreja. Se observó también en los casos positivos un mayor número de glóbulos rojos parasitados en el borde de la lámina. En animales con infección mixta de especies Babesia y A. marginale, se observó mas A. marginale cuando el frotis se preparó con sangre tomada de la yugular. Los métodos de enriquecimiento brindaron alguna seguridad ya que se detectaron parasitemias bajas que no se vieron haciendo el frotis cuando se agitó la muestra de sangre. En parasitemias altas con los métodos de enriquecimiento hubo una mayor concentración de parásitos en el frotis, con porcentaje de 1-2 para el método de enriquecimiento y de 0.5% en frotis preparados agitando la sangre. Después de unas 3 horas de estar en reposo la sangre, se obtuvieron resultados diferentes al hacer los frotis de la parte superior del contenido del tubo, de la mitad y del fondo.

En este caso los resultados fueron del 1-2% para el fondo, 0.5% para la mitad y menos 0.1-0% para la parte superior del tubo.

De los anticoagulantes, el mejor fue el EDTA porque conservó el tamaño y morfología de las células rojas un mayor tiempo a medio ambiente. La combinación de oxalato de sodio y potasio resultó también bastante buena y no hubo substanciales variaciones en la morfología y tamaño de las células. El citrato de sodio en cambio, rompió gran número de glóbulos después de 8 horas de tomada la muestra de sangre.

En muestras conservada al medio ambiente, se observaron muchos glóbulos crenados después de 24 horas y los parásitos aunque se vieron, se presentaron algo deformados. De las muestras conservadas en refrigeración ninguna fue parasitada pero en 7 días hubo pocas modificaciones en la morfología celular. La coloración en estas muestras iba tomando cada día un tánte mas azul. El pH para preparar el colorante que funcionó mejor fue del fosfato buffer monobásico de pH 7.3 que coloreó el núcleo del parásito de un rojo cereza y el citoplasma de azul claro. La intensidad de la coloración aumentó a medida que el pH se iba haciendo mas ácido, con el inconveniente de una poca adherencia del frotis a la lámina, que muchas veces se desprendió al lavado y problemas de precipitación que impidieron observarlos. El tiempo de exposición del colorante por 25-30 minutos resultó satisfactorio (Figura 11).

Conclusiones:

Teniendo en cuenta la mayor fragilidad de los glóbulos parasitados recomendamos que los frotis sean hechos en el menor tiempo posible de tomada la muestra. Cuando esta tenga que soportar variaciones de temperatura a través de distancia y tiempo, aconsejamos protegerla mediante refrigeración o efectuar el frotis y fijarlo con metanol en el lugar donde se colecta. La fijación es un punto importante para una buena coloración, debe hacerse con alcohol metílico absoluto que debe estar bien tapado, por su propiedad higroscópica. Los frotis fijados que no vayan a colorearse inmediatamente pueden mantenerse en congelación por muchos días sin que se observen modificaciones en la tinción y en la morfología de las células y de los parásitos. Como los eritrocitos parasitados son mas propensos a la lisis, las coloraciones deben ser preparadas con soluciones buferadas con pH que fluctúe entre 7.2 y 7.8.

Los frotis hechos con intervalos de tiempo durante 24 horas, de muestras poco parasitadas conservadas al medio ambiente y tomadas con EDTA y oxalato de amonio y potasio, no mostraron cambios morfológicos marcados en la célula, solo cambios de morfología en el parásito. En muestras muy parasitadas, los eritrocitos se deforman mas pronto y los parásitos se destruyen en un tiempo relativamente corto. En muestras muy parasitadas después de 24 horas en medio ambiente, se observan restos del parásito en glóbulos muy deformados. En refrigeración, las muestras se conservan mucho

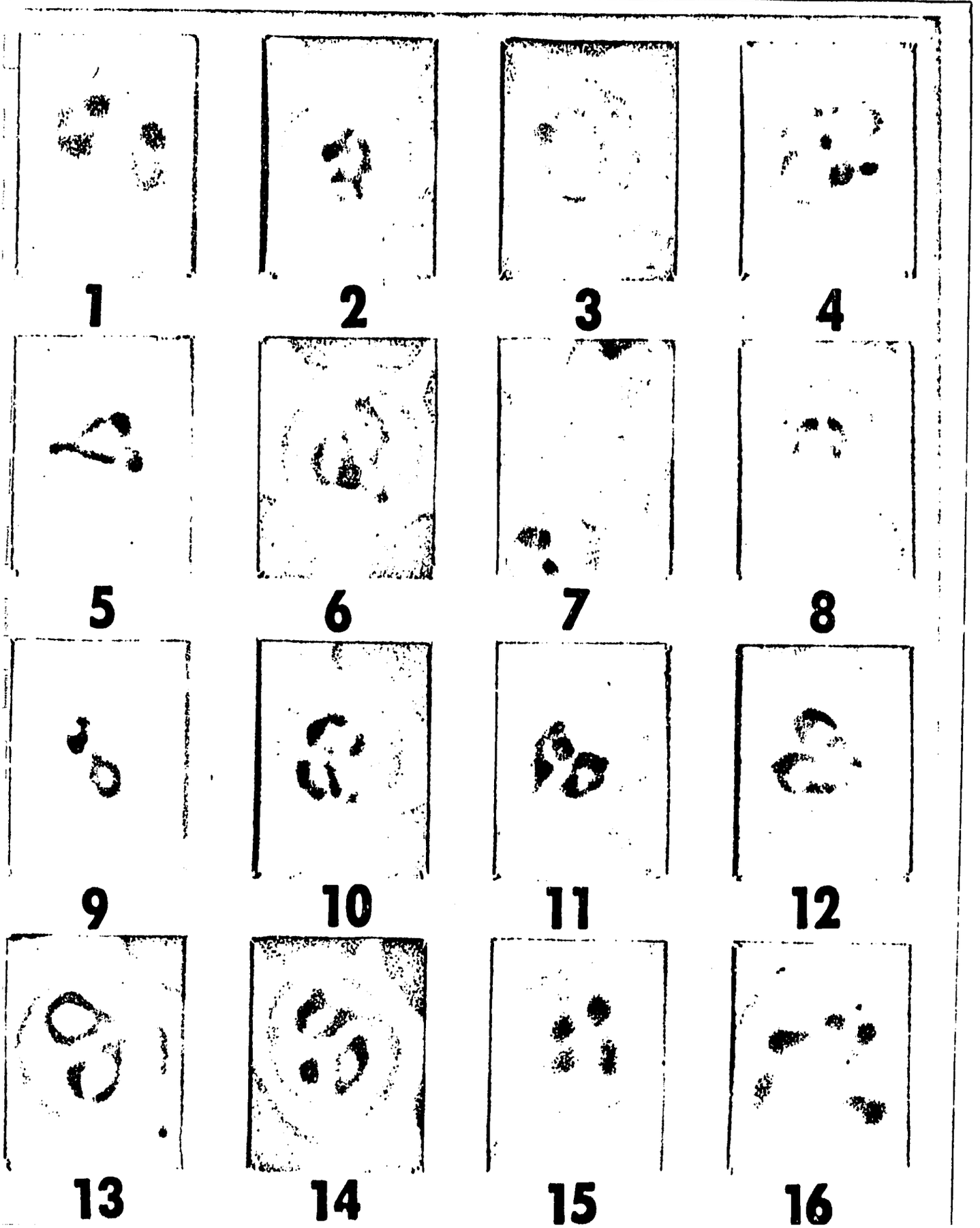
tiempo. El citrato de sodio es un anticoagulante bueno si las muestras se procesan poco tiempo después de tomadas.

Un baja parasitemia no siempre es indicativa de la bondad de la enfermedad porque parece que hay cepas que tienen infinidad de órganos internos produciendo graves perturbaciones sin que se manifieste el parásito en la sangre circulante. En este caso, pruebas serológicas de fijación del complemento, de precipitación en gel y otras deben acompañar a un frotis negativo de muestras que provengan de animales con clásica sintomatología de una babesiosis.

Las especies de Babesia tienen también mayor afinidad por la sangre periférica en la cual observamos casos positivos cuando apenas comezaba el incremento térmico. Cuando el frotis se hizo a partir de métodos de enriquecimiento en un tiempo no mayor de 2 horas, los resultados fueron buenos. Con un tiempo mayor, los eritrocitos parasitados se concentraron en el fondo del tubo. Se piensa que, procesos metabólicos en el parásito o cambios celulares o un intercambio iónico modifiquen el peso específico de estos glóbulos. Las soluciones para preparar el colorante no deben tener cloruro de sodio porque hay posiblemente remoción de la hemoglobina que influye para que el frotis no se adhiera y caiga al lavado o se inhibe completamente la coloración. La intensidad de la coloración además de los cambios celulares y del parásito parece depender del pH mas que de cualquier otro factor.

Nosotros continuaremos trabajando en nuestro laboratorio hasta que el completeo desarrollo de esta técnica pueda servir a la investigación de la babesiosis bovina.

Fig. 11. Photomicrographs of peripheral blood films of cattle acutely infected with *Babesia bigemina* and *Babesia argentina*. Stained by Giemsa stain. X1000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6. Cross-Immunity of Babesia Infections in Colombia

Introduction:

The importance of the immunological differences between strains of hemoprotozoal parasites in the epidemiology of human malaria is well known (Taliaferro, 1948). A disease comparable in some aspects with malaria is bovine babesiosis. Babesiosis is transmitted by an arthropod (tick) and the protozoan parasite which causes the disease invades the red blood cells. In Australia, it has frequently been observed that some cattle when moved from one locality to another within the enzootic babesiosis areas, show clinical signs of Babesia infection. Therefore, the suggestion has been made that different strains of the parasites differ in immunity produced or in virulence (Riek, 1965). In South America, Miranda and Horta (1913) reported that animals premunized against Babesia bigemina and Babesia argentina contacted a fatal attack of babesiosis after arrival in Brazil. Further study of Babesia strain immunity is obviously needed in Colombia.

The early work of Theiler, McFadyean and Stockman, Lignieres and Brumpt demonstrated that animals which have recovered from an infection with one species of Babesia may be infected with another species either by bite of an infected tick or by direct inoculation of infected blood. Immunological tests help to separate different species, but on the other hand, different strains of a single species may not yield similar results. In this respect, Rosenbusch

and González (1924) showed that a cattle disease (tristeza) in Brazil was caused by a parasite corresponding with B. bigemina. It was found that parasites from different localities vary immunologically. Cattle which had recovered from an acute infection caused by a strain from only one locality were immune to this particular strain, but they could be infected with strains from other localities. Equally, Babesia canis from dogs of French origin did not provide immunity for these hosts against the North African strain, which is morphologically indistinguishable from it. Du Toit (1918) included Babesia divergens as a species of this subgenus Babesia, but Wenyon (1926), Donatien and Lestoquard (1930) and Neitz (1956) considered Babesia divergens to be a synonym of Babesia bovis. Until the status of these Babesia is more clearly understood, it is obvious that more research is needed on the strain immunity of Babesia parasites.

Objectives:

To determine the degree of strain immunity of Babesia parasites isolated from the infected cattle located in the Valle del Cauca, the Northern Coast and Llanos in Colombia. To compare the degree of sterile and co-infectious immunity in respect to strain immunity by cross-immunity experiments.

Materials and Methods:

Twenty intact and 10 splenectomized Holstein-Friesian male calves of the same age were inoculated with different isolates of

Babesia bigemina and Babesia argentina respectively. Blood from these animals was collected in citrate solution and used for determination of percent parasitemia and hematocrit values. The source of inoculum was brought to the L.I.M.V.from the animals infected with babesiosis located in Valle del Cauca, Northern Coast and Los Llanos. During the acute stage of disease, the following parameters were recorded: temperature, parasitemia, packed cell volume, antibody titers and mortality. The animals which recovered the primary attack were challenged with homologous and heterologous original strains of Babesia parasites. The second group of 10 intact and 10 splenectomized Holstein-Friesian male calves of the same age were vaccinated with killed-Babesia vaccine, prepared from the killed Babesia parasites isolated from infected animals which were located in different regions in Colombia. Response to vaccination was determined by hematocrit (H), parasitemia (P) and antibody titer (AT). Approximately 2 months after vaccination, the animals were challenged with blood-borne Babesia of homologous and heterologous original Babesia strains. Response after challenge of vaccinated animals was determined by parasitemia (P). packed cell volume (PCV), mortality (M) and antibody titer (AT). The data will be analyzed statistically in order to confirm the reactions to the challenge of different strainst of Babesia parasites.

Results:

The preliminary results of these experiments are shown on

Table 1. In general, it was found that a certain strain of the same Babesia spp. lacks cross immunity. However, more research is needed to confirm these observations. It was found that animals recovered from Babesia infection caused by mixed infection of Babesia bigemina, Babesia argentina and Babesia major from the Northern Coast (Montería, Turipaná) were fully susceptible to the blood-borne challenge of Babesia bigemina and Babesia argentina from los Llanos. One particular animal (No. 609) had a parasitemia of 40% and finally died with acute babesiosis. A second animal (No. 1869) recovered from the Babesia infection caused by Babesia bigemina from Río Magdalena and was fully susceptible to infection with Babesia bigemina and Babesia argentina from Los Llanos. This animal had a parasitemia of 1%, but survived the blood-borne challenge. A third animal (No. 3568) recovered from an infection with Babesia argentina isolated from the Valle del Cauca and was fully susceptible to the Llanos strain of Babesia bigemina and Babesia argentina. This animal died with acute symptoms of babesiosis manifested with a parasitemia of 12%.

Results and Conclusions:

Because these experiments are not completely finished, no specific conclusions can be made on cross immunity on the basis of the results reported herein. But, some very interesting findings can be discussed. The lack of cross immunity observed should not be accepted as a final finding because all the animals inoculated

TABLE 1. Preliminary Results on Cross Immunity Between Different Strains of *Babesia* Parasites.

Recovered from *Babesia* spp.	Challenged with *Babesia* spp. (Llanos Orientales) # 21 and # 3869
Animal # 609 - *Babesia major* Turipaná, Montería (North Coast)	Parasitemia 40% and Death *Babesia bigemina* *Babesia argentina*
Animal # 1869 - *Babesia bigemina* Río Magdalena	Parasitemia 1% *Babesia bigemina* *Babesia argentina*
Animal # 3569 - *Babesia argentina* Valle del Cauca	Parasitemia 12% *Babesia bigemina*

with blood from the three different locations were chemotherapeutically treated with the new babesiacidal drug 4A65. The drug 4A65 was injected to prevent death loss in these infected animals. The possibility exists that these animals were sterilized after the treatment with this drug may have been fully susceptible to further challenge with Babesia spp.

Another possibility is that these animals were not sterilized and that no crossed immunity was produced. This discussion is without experimental support. Experiments currently in progress will hopefully answer some questions raised here.

The solution of the problems of cross immunity must be found before an effective vaccination program can be devised.

7. Protección Mixta por Vacunación contra Babesiosis y Anaplasmosis y Control de Parásitos Gastrointestinales.

Introducción:

En los primeros experimentos realizados en Palmira C.N.I.A. encontramos que animales vacunados contra babesiosis no eran protegidos contra anaplasmosis, muriendo por esta causa y viceversa. También y debido a la exposición natural a todo tipo de parásitos los animales se infestaban por parásitos gastrointestinales muriendo por esta causa.

En el experimento que se proyecta se trata de obviar las causas de mortalidad anteriores a fin de obtener una protección mixta contra

anaplasmosis y babesiosis al mismo tiempo que se llevará un estricto control de los parásitos gastrointestinales.

Material y Métodos:

Fueron seleccionados 20 terneros de raza Holstein de 4-6 meses de edad los cuales se dividieron en 4 grupos. El experimento se inició el 4 de septiembre de 1969, recolectando sangre para hacer controles iniciales de parasitemia, hematocrito y test para serología en Anaplasma y Babesia. El mismo día fueron pesados y se recolectó también material fecal para conocer el estado de infestación por parásitos gastrointestinales.

El chequeo coproparasitario se realizó el día 5 de septiembre de 1969; en vista de los resultados se procedió a vermifugarlos con Ripercol a la dosis de 1 cc X 5 libras de peso, de la solución preparada disolviendo in sobre de 62.5 gramos en 1000 cc de agua. Estratamiento se realizó el día 11 de septiembre de 1969.

El chequeo coproparasitario se seguirá efectuando cada 30 días y de acuerdo con los resultados se realizarán nuevas vermifugaciones. En 23-IX-69, todos los animales fueron divididos en grupos A y B.

-VACCINE - AG-S + Bacto-Adjuvant Complete

GROUP A

-PREMUNITION - *Anaplasma marginale*

No.	Animals	Type of Immunity
1	6923	-Sterile *Babesia*
2	6926	-Co-infection *Anaplasma*
3	6930	
4	6931	
5	6932	
6	6955	
7	6959	
8	6960	
9	6961	
10	6962	

Experimental Design

1. Examination for Intestinal Parasites (September 5, 1969)
2. Treatment against Helminths (September 11, 1969)
3. Premunition -*Anaplasma marginale* 10 ml. of blood (#8) in EDTA, s/c (September 23, 1969)
4. I. Vaccination with AG-S 10 ml. of AG-S + Adjuvant (aa) s/c
5. II. Vaccination with AG-S 10 ml. of AG-S + Adjuvant (aa) s/c (October 7, 1969)
6. Treatment with 356-C-61 (Anaplasmosis) 4 mg/kg IV (October 14, 1969)
7. Response to Vaccination (4 weeks)
8. Tick-borne challenge with *Boophilus microplus* (October 26, 1969)
9. Response to challenge (H, AT, T, P)

Estos animales fueron premunizados contra anaplasmosis inyectando 10 cc de sangre provenientes de animales portadores esplenectomizados en el Laboratorio de Investigaciones Médicas Veterinarias (LIMV) de Bogotá. La sangre fue recolectada en EDTA por vía subcutánea.

I. Al mismo tiempo los animales fueron inoculados con 10 cc de Babesia AG-S mas 10 cc de diluyente comercial oleoso H 37-Ra también por vía subcutánea.

II. A los 14 días (7-X-69) se repitió la vacuna AG-S con la misma dosis y vía que la vacuna utilizada antes. En 14-X-69 se hizo tratamiento contra Anaplasma con Compound 356-C-61 inyectando 4 mgrms./kgrms. de peso vivo por vía intravenosa lentamente.

GROUP B	-PREMUNITION - Babesia bigemina and Babesia argentina -PREMUNITION - Anaplasma marginale	
No.	Animals	Type of Immunity - Co-infections
		Experiment Design
1	6936	1. Examination for intestinal parasites (September 5, 1969)
2	6939	
3	6941	2. Treatment against Helminths (September 11, 1969)
4	6944	3. Premunition - Babesia bigemina and Babesia argentina - 10 ml. blood
5	6945	
6	6947	(#3569 and #3868 aa in EDTA s/c) (September 23, 1969)
7	6949	4. Premunition - Anaplasma marginale 10 ml. of blood (#8) in EDTA s/c
8	6951	(September 23, 1969)
9	6952	5. Treatment with 4A65 (Babesia) 2 mg - IM/kg
10	6954	(September 30, 1969)
		6. Treatment with 356-C-61 (Anaplasma) 4 mg/kg IV (October 14, 1969)
		7. Response to Premunition (4 weeks)
		8. Tick-borne challenge
		9 Response to challenge (H, AT, T, P)

Este grupo fue también premunizado contra Anaplasma utilizando la misma técnica que para el grupo A e inyectando droga en la misma fecha (14-X-69) contra anaplasmosis. Este grupo fue premunizado contra B. bigemina y B. argentina; la sangre se recolectó en EDTA de portadores esplenectomizados en el LIMV de Bogotá. Se utilizaron 10 cc de sangre mas 10 cc de diluyente al igual que para el grupo A, vacunando por vía subcutánea. Los animales de este grupo se trataron en 30-IX-69 contra babesiosis con droga experimental 4A65, utilizando 2 mgrms por vía intramuscular. Se hicieron colecciones de sangre en tubos, unos con EDTA para conocer parasitemia y hematocrito y otros para obtener suero a fin de realizar pruebas para anaplasmosis y babesiosis dos veces por semana. En esta forma estuvieron confinados hat 26-X-69 sin ser expuestos a parásitos externos a fin de lograr resistencia. En 26-X-69 salieron a pastoreo donde se conoce hay garrapatas y se hicieron tomas de sangre una vez por semana durante 5 meses para conocer el estado de parasitemia y para colección de garrapatas para su identificación. Manejo y alimentación de los animales se hizo durante el primer mes con ensilaje de maíz, caña y concentrado comercial (perpartina). En el período de exposición la alimentación fue a base de pasto y concentrado comercial (cría-vaquina). Fecha de iniciación del experimento: Septiembre 4, 1969. Duración Probable: 6-8 meses.

Results and Conclusions:

The data of this experiment are not available at the present

time since these animals are under field exposure to babesiosis and anaplasmosis. However, data on fecal examinations from 20 animals used on vaccination trials are available and are recorded. All 20 animals were found to be infected with helminthic parasites. Degree of infection was from slight to heavy. The following species were identified: Haemonchus, Trichostrongylus, Ostertagia, Cooperia, Oesophagostomum, Trichuris, Monezia and Eimeria. On the basis of this observation, the conclusion can be made that an effective program against hemoparasitic infection cannot be succesfully conducted without taking into consideration the degree of helminthic infection. Helminthic infection will interfere with the degree of immunity produced by the host vaccinated with hemoprotozoal vaccines.

This approach to the control of helminthic infections will have significance in devising control programs against hemoparasitic diseases in Colombia.

8. Collection and Identification of Tick-vectors in Colombia

Introduction:

In view of the great potentialities of a large number of tick species for transmitting disease pathogens in all parts of the world, the necessity of their control requires little or no elaboration. In Colombia, the tick-borne diseases caused by Babesia parasites and transmitted by certain species of ticks are, and have been, long responsible for a much higher proportion of the very high animal

loses than was previously recognized. In order that control measures can be properly used and the control of ticks successfully accomplished, it is necessary to know the exact distribution and population of tick vectors involved in the epidemiology of bovine babesiosis. At the present time, there is not enough information in respect to the population and distribution of tick vectors in Colombia. Therefore, some attempts were made to survey the tick population in certain parts of Colombia, particularly where the incidence of babesiosis is highly prevalent.

Objectives:

To collect and identify tick species that may be involved in the epizootiology of bovine babesiosis. To determine the effect of environmental conditions on the tick populations and to survey the incidence of tick infestation on certain breeds of cattle.

Material and Methods:

Animals infected with Babesia bigemina and Babesia argentina were used as a source of the tick collections from Valle del Cauca, Río Magdalena and Costa Norte. Ticks were collected from the different parts of the animal body and preserved in absolute alcohol. Microscopic examination of the peripheral blood smears from animals infected with Babesia was made to establish infections. The collection of ticks was made throughout the year. Adults, nymphs and larvae of both sexes were collected

Results and Conclusions:

In January, 1969, ticks were collected from 10 animals at the ICA farm in Palmira. These animals were known to be infected with Babesia bigemina and Babesia argentina. The species of ticks were identified at the Parasitology laboratory at the National University in Bogotá as Boophilus microplus including 7 male and 31 female adults. During February and March, 1969, the following ticks were collected from the same animals: Boophilus microplus: 10 females, 10 males and 57 nymphs; Anocentor nitens: 21 females, 15 males and 29 nymphs. In May, 1969, on 2 private farms in the Magdalena River Valley, 1 female Amblyomma cajennense and 3 females and 2 nymphs of Boophilus microplus were found.

On ICA and private farms in Valle del Cauca, the following species of ticks were identified during September, 1969: 35 female, 6 males and 17 nymphs of Boophilus microplus; 49 females, 72 males and 18 nymphs of Anocentor nitens. On October, 1969, on the ICA experimental farm in Palmira there were 109 females, 146 males and 161 nymphs of Anocentor nitens. On the Magdalena River Valley 3 female of Boophilus microplus were found in November, 1969 and in the Fusagasugá area in the same month 2 females identified as Anocentor nitens were found. Also in November, 1969, at Bugalagrande, Valle del Cauca, 19 males, 10 females and 62 nymphs of Anocentor nitens and 2 females of Boophilus microplus were identified from Hacienda San Marcos. During the month of December, collections were again made near Girardot and Hacienda Lucerna and 75 males, 380 females and 155 nymphs of Boophilus microplus were identified.

From this survey, very interesting observations can be made. Boophilus microplus were present in the enzootic areas of babesiosis and anaplasmosis. This confirms previously published reports that this vector was involved in the epizootiology of infections of Babesia spp. in Colombia. However, the fact that Anocentor nitens were found on animals known to be infected with babesiosis and anaplasmosis is a very interesting finding and the significance of this is not evident. It is well known that Anocentor nitens has as its host the horse, and it has been reported as a vector of equine babesiosis in the United States. The question remains is the bovine an accidental host of Anocentor nitens and, if not, what is the role of this tick in the transmission of bovine babesiosis? These questions must be answered by additional experimental work to clarify this relationship before a control program of tick vectors can be designed.

9. Isolation of Babesia Antigens for Use in Serologic Tests

Introduction:

Ever since babesiosis was first recognized (Babes, 1888), veterinarians have felt a growing need for a practical method of diagnosis. The nature of babesiosis is such that the acute stage of the disease is accompanied by anemia. The diagnosis of the acute phase of the disease has usually been made by examination of stained blood smears. However, animals that

recover from acute infection later become carriers and cannot be differentiated clinically from non-infected animals. In such animals, Babesia can seldom be found in stained blood smears.

Objectives:

To evaluate the various serologic techniques for detection of Babesia antibodies (1) complement fixation tests, (2) tube latex agglutination (3) hemagglutination and (4) gel precipitation techniques. To develop a practical serologic test for the diagnosis of babesiosis in Colombia, and to accomplish prevalence surveys of different climatic zones in Colombia.

Material and Methods:

A known strain of Babesia bigemina was used for infecting calves and for producing Babesia bigemina antigen. A known strain of Babesia argentina was a so for producing Babesia argentina antigen. A strain of Babesia spp. (LIMV) was used for producing Babesia spp. antigen.

Antigen was prepared from blood in which at least 25-30% of the RBC are parasitized. High parasitemias were developed by serial passage of the parasites through 3-5 splenectomized calves. During the course of each clinical attack following blood passage, thin smears of peripheral blood were taken every 3-4 hours to determine the proportion of parasitized RBCs as indicated by little or no change on two successive examinations. When the parasitemia remained

constant, the organisms were passaged to the next animal. After the 3rd, or 4th passage with B. bigemina, up to 46% RBCs were parasitized, and with B. argentina infection up to 30% RBCs were parasitized.

Blood for the preparation of antigen is drawn into flasks containing EDTA (ethylenediamine tetraacetic acid) from calves with 25-40% parasitemia, (EDTA - 1.2 g. per liter of blood). Plasma was removed by centrifugating 2,500 r.p.m. for 20 minutes. RBCs were washed twice in 0.85% NaCl. The volume of packed RBCs was measured and ten times this volume of H_2O distilled at 4-5°C. was added. Lysis was allowed to proceed for one (1) hour at 3-5°C. Then, the material was centrifuged at 3,500 x g. for 30 minutes (in cold 4-6°C) Sorvall. After centrifugation, the supernatant was discarded and the sediment was resuspended and homogenized in isotonic Veronal Buffer and used as the antigen. This mixture was frozen rapidly to -79°C in 2 ml. amounts (preserved in this way it retains its antigenity for at least 6 months). On the day of the test, the mixture was thawed rapidly by immersing the ampoules in a water bath at 37°C. To prepare the working antigen, an equal volume of cold Veronal buffer pH 7,2 was added and homogenized on the day of the test. This solution was centrifuged at 27,000 x g. for 30 minutes in a Sorvall RC2B refrigerated centrifuge and the supernatant fluid was used as the antigen. This process can be repeated as long as antigenic activity is present in the sediment. Control antigen was prepared in the same manner from

Complement titration and preparation of hemolytic systems were made according to the "Manual for Conducting the Complement Fixation test for Anaplasmosis." The agglutination and gel precipitation Tests were performed according to the technique by Todorovic et. al. (1967).

Results and Conclusions:

Babesia antigens were isolated and successfully used in Complement Fixation, agglutination and gel precipitation techniques. Antigens were found specific to detect corresponding Babesia antibodies. If preserved at low temperatures, these antigens were stable for up to 6 months. Antigens were derived from 2 sources in the blood during the acute infection with Babesia spp. First, it occurs in the infected erythrocytes and second in the plasma or serum. The specificity of this antigen was confirmed by testing known positive sera and by producing specific antibodies against the antigen by injecting chickens. The antiserum from the chickens was collected and tested with the double gel diffusion precipitation procedure.

In well designed experiments, it was found by the CF test that the first detectable antibodies appear in serum 8-9 days post-inoculation and persist for several months.

Further experiments are needed to define the nature of these antigens. Do they consist of proteins, lipoproteins, lipids, polysaccharides or others? This can be determined by biochemical

methods and on the basis of these findings better methods for their preservation can be devised. Can these antigens be used to produce immunity, and what kind of a role do they play during an infection? Although these antigens have been succesfully used for the serologic diagnosis of babesiosis, further experimental work is needed to develop a more practical sensitive and accurate test for diagnosis of babesiosis in Colombia.

B. Babesiosis Projected Projects

1. Immune Response of Cattle Vaccinated with Irradiatide Attonuated Babesia, Anaplasma and Trypanosoma Vaccines, in Colombia.

Introduction:

Parasitic diseases represent serious problems to animals and man, particularly in the developing areas in the world. In Colombia, a high incidence of parasitic diseases is reported in the field of veterinary and human medicine. In view of the important role that ionizing radiation and radio-isotopes play in the production and utilization of vaccines against such diseases, and in view of the probable importance of immunity and immuno-pathology in parasitic diseases, emphasis on research in this area is needed.

The present situation in regard to the use and development of attenuated vaccines against helminthic and protozoal diseases according to the data available in literature is as follows: Irradiation of hemotropic and other parasites interferes with

their physiological processes and frequently inhibits their normal development and multiplication and this consequently leads to the attenuation of their virulence. Although ionizing irradiation inhibits avian malaria parasites in their normal multiplication, Bennison and Coatney (1945), Rigdon and Rudisell (1945): it has reported that the reproductive processes are affected by a lower dose of radiation than the metabolic processes, Ceithaml and Evans (1946). Therefore, the use of irradiated parasites in vaccine might enable one to take advantage of the special immunological properties of living parasites while at the same time suppressing the pathogenic effects of the vaccine resulting from the reproductive capacity of the parasite involved.

Objectives:

1. To investigate the possibility of the production of radiation attenuated parasitic vaccine already in commercial use in other countries.
2. To investigate the effect of irradiation on the attenuation of Babesia, Trypanosoma parasites for use in vaccines.
3. To develop a cooperative research project with (1) Instituto Nacional de Cancerologia, (2) Instituto Colombiano Agropecuario and (3) Texas A&M University.

Review of Present Status:

The present situation in regard to the use and development of

vaccines against helminthic and protozoal diseases is summarized as follows: (1) vaccines in commercial use, (2) vaccines with promising commercial applications, (3) vaccines with encouraging laboratory results and (4) vaccines meriting further investigation.

Lungworms: (Vaccine in commercial use) - The two lungworms Dictyocaulus viviparus and Dictyocaulus filaria vaccines are gaining increasing acceptance in agricultural practice. Hookworms: (Vaccine with promising commercial use). An irradiated vaccine for hookworm disease of dogs is in an advanced sted of development and is likely to be marketed in the near future. It is thought that consideration should be given to the development of human hookworm vaccine (Ancylostoma duodenale and Necator americanus) using monkeys as laboratory hosts. Because of its significance in human health, this project should be considered in conjuction between human and veterinary medicine research laboratories. Gapeworm (Syngamus trachea) (Vaccine with encouraging laboratory results). Laboratory experiments and preliminary field trials have given promising results by using irradiated vaccine. With respect to the problem of vaccination of day-old chicks, turkeys and game birds, and the technical problems of distribution of vaccine, it is suggested that vaccination in the hat hery would offer a practical solution. It is recommended that this work should be encouraged and continued. Echinococcus (Vaccine with encouraging laboratory results). In view of the zoonotic significance of Echinococcus granulosos in many countries (e.g. the Mediterranean litoral, Australia, New

Zealand, Americas, Asia) and the results of preliminary experiments in which up to 92% protection has been achieved in dogs following vaccination with irradiated protoscoli s, it is recommended that this work also be encouraged and continued. Schistosomes (Meriting further) A considerable volume of knowledge has been accumulated from experimental infection of various laboratory hosts with irradiated Schistosoma mansoni and S. japonicum and although the results do not currently justify the application of this technique to the field of human vaccination, further research on this group of parasites should be encouraged with a view to investigating basic immunologic phenomena. Liver fluke (Fasciola spp.) (Meriting further study). Because of the widespread distribution and economic significance of these parasites, the possibility of vaccination using irradiation should continue to be explored as part of a general co-ordinated program. Haemonchus and other gastrointestinal nematodes (Meriting further study). The major problem in the development of a satisfactory vaccine to Haemochus contortus appears to be the prolonged unresponsive period in lambs. This is of considerable immunological interest and irradiated vaccine may be of use as a tool in this study.

PROTOZOA: Malaria (Plasmodium) (Encouraging laboratory results). The preliminary results in mice are encouraging and further expanded study is recommended. Trypanosoma, Anaplasma, Babesia (Meriting further study). Irradiation studies should be encouraged in view of their potential value in the development of vaccines and as an

immunological tool. Some encouraging results have been obtained by irradiation of Anaplasma marginale in the United States. Theileria spp. (Meriting further study). East Coast Fever is a serious problem in Central and East Africa. Neither chemotherapy nor vaccination is available and prophylaxis depends entirely on vector control. X-irradiation vaccines should be considered in this field.

Material and Methods:

A gammacell 220 cobalt-60 irradiator, described by Rice and Smythe (1960) will be used as the source of gamma radiation. This 1300 Ci source will deliver a dose-rate calculated by the Friecke ferrous sulphate method of Weiss et. al. (1955) at approximately 900 R/minutes. The tubes containing the blood will be placed in a circular rack. A planetary gear system will permit the test tube holder to rotate on its own while the test tubes will be rotated on their axes.

This method will insure a uniform exposure to radiation of the red blood cells in each of the test tubes. The injected blood will be kept in an ice bath until the time it will be placed in the irradiator and afterwards until the animals will be injected.

Control immunization will be made using (1) saline citrate solution, (2) irradiated normal bovine blood and (3) supernatant from irradiated parasitized blood. Irradiated blood will be centrifuged for 20 minutes at 2000 r.p.m. and the plasma will be drawn off. Then plasma will be inoculated through 13 mm. 0.45 milli-

microns millipore Swinney-13 filters.

A first series of preliminary experiments will be undertaken to determine the effect of exposure to gamma radiation on the ability of Babesia, Anaplasma, Trypanosoma respectively to establish an infection in young calves. A total of 120 calves, Holstein-Friesian, 2-5 months of age will be separated into 12 froups. The calves in the first group will receive parasitized blood exposed exposed to 10,000 R. Those of the other groups will receive approximately the same number of parasitized red blood cells which had been exposed to different doses of radiation varying from 10,000 R. to 60,000 R, respectively.

Animals will be examined for the presence of Babesia, Anaplasma and Trypanosoma organisms, respectively, which will be determined by thin and thick blood smears stained with Giemsa stain. In addition to the blood examinations, complement fixation tests will be used for detecting the corresponding antibodies in sera of the vaccinated animals. The degree of immunity will be determined by blood-borne and tick-borne challenge.

2. Demostración de Inmunidad Pasiva contra Babesia bigemina por Inoculación de Suero de Animal Recuperado de Babesia bigemina

Introducción:

Cuando un animal se recupera de una infección aguda con Babesia bigemina, este animal es resistente a una nueva infección. La pre-

gunta es por qué mecanismo se opera esta inmunidad, (1) por anticuerpos humorales o (2) por inmunidad celular. Si la inmunidad contra Babesia es básica con anticuerpos humorales, hay la posibilidad de transferir sueros de animal recuperado y demostrar la inmunidad pasiva.

Objectives:

Demostrar la inmunidad pasiva de la babesiosis. Determinar la posibilidad de efecto terapeutico del suero de animales recuperados de Babesia bigemina y Babesia argentina.

Material y Métodos:

Todos los animales recuperados de Babesia bigemina serán sangrados (10 ml. de sangre). Al día siguiente se obtendrán los sueros para efectuar la fijación del complemento con el objeto de saber por qué tienen un título alto. Se sangrarán los animales con título alto (1 litro de sangre) y el suero será separado. PARTE #1. Profilaxis contra Babesia bigemina. Se inyectará un animal normal con suero inmune por vía intramuscular (1 ml./kg. de peso). Una semana después serán inyectados 20 ml. de sangre por vía intramuscular que habrá sido tomada en EDTA e infectada con Babesia bigemina. Se inoculará un animal con suero inmune (1 ml./kg de peso) y al mismo tiempo 20 ml. de sangre infectada con Babesia bigemina tomada en EDTA por vía intramuscular. Diariamente estos animales serán sangrados; se harán

láminas, hematocritos así como colección de los sueros y la temperatura durante 3 semanas. Si el suero inmune tiene anticuerpos, no habrá parasitemia. PARTE # 2. Tratamiento curativo contra Babesia bigemina. En este caso, el suero animal con un alto título contra Babesia bigemina será refrigerado (500 ml. de suero en botellas plásticas). Si hay un animal que padece una babesiosis aguda, fiebre alta, parasitemia, hematocrito bajo, etc., este animal será inoculado con suero inmune (1 ml./kg de peso). Si existe alguna protección en el suero inmune contra Babesia bigemina, el animal con síntomas agudos tendrá una fase resolutiva favorable. Si por el contrario, el suero inmune no tiene ninguna protección, el animal continuará con la enfermedad hasta morir.

3. Culture of Babesia parasites in Vitro

Introduction:

The simple technique of growing one or two generations of malaria parasites in a test tube as described by Bass and Johns (1912), has been modified successively by Siton (1912), Thomson and McLellan (1912), Zieman (1915), Row (1917) and Ioff (1925) and most recently for the cultivation of avian malaria parasites by Manwell and Hewitt (1937). Although the method has been much simplified,it has failed in its main object, i.e. to cause the parasites to multiply and survive. However, Garnham (1966) has

found that this modified technique gives excellent results in the first 24 hours of growth of Plasmodium falciparum and Plasmodium coatney; after that, there is little further development and the schizont become abnormal in appearance. Because of simplicity of the method described for the cultivation of malarial parasites an attempt will be made to grow Babesia parasites by using the same technique.

Objectives:

To develop a technique for cultivation of Babesia bigemina and Babesia argentina in vitro. To determine the effect of different cultures on the infectivity of Babesia parasites.

Material and Methods:

With sterile precautions, place 7 to 10 ml. venous blood (Babesia bigemina) into a test tube which contains a thick wire with a loop at the lower end. The other end projects through the cotton wool plug (or gauze) and is rapidly rotated in order to defibirnate the blood. The fibrin is then withdrawn with the wire and a sterile plug inserted. Add four drops of 50% solution of dextrose sterilized by autoclaving or sterile Alsever's solution. The defibirnated blood is cultured at 37°C, the erythrocytes quickly become sedimented and are separated from the clear plasma by the buffy coat. The parasites should grow principally in the erythrocytes immediately beneath the latter layer. With a fine pipette (Pasteur) remove at appropriate intervals (every hour)

a small drop of the upper layer of erythrocytes mixing it with a little plasma on withdrawal. A thin blood film is made and stained by Giemsa. NOTE: If this technique fails to give good results, try culture mediums: Harward and Trager's methods of culturing Plasmodium lophurae freed from erythrocytes.

4. Diagnosis of Bovine Babesiosis in Tissue Smears

Introduction:

Bovine babesiosis is a common disease of tropical and subtropical areas. Its diagnosis is commonly made by blood smears, but many factors such as pyremic periods, conditions of a chronic phase of the disease, and some species of Babesia which present a low parasitemia impede the diagnosis by smears made from circulating blood. It is thought that Babesia, or some Babesia species, in certain periods of evolution have some affinity with determined tissues such as the spleen, liver, kidneys, ganglions, bone marrow, etc. These tissues frequently arrive at the diagnostic laboratory many hours after the animal's death. Keeping in mind the destruction of parasites, the diagnosis by tissue smears is limited to a determined period of time on which this study is based.

Objectives:

To compare the percent parasitemia by smears made from blood taken from the jugular vein and different tissue sections. To

observe when the destruction of parasites starts in the blood and tissues after death. To find out which Babesia species has more affinity for certain internal organs.

Material and Methods:

A total of 5 animals will be inoculated with blood containing Babesia bigemina and Babesia argentina and with strains from Los Llanos, Valle del Cauca and the Costa Norte. When an increase in temperature and parasitemia starts, smears prepared from the blood of the jugular vein, the tail and the ear will be made. Immediately, the animal will be euthanatized to obtain smears from the different tissues, these will be made immediately, then after 5 minutes, 10 minutes, 15 minutes.....and until the destruction of parasites begins. PART # 1. An animal with low parasitemia will be killed. PART # 2. Two animals with high parasitemia (10-20%) will be killed. PART # 3. Two convalescent animals with negative results in smears made from blood of the jugular vein, tail and ear will be killed.

5. Babesia bigemina and Babesia argentina Transmission Experiments Using Boophilus microplus and Anocentor nitens.

Introduction:

In order to appreciate the techniques by which the Babesia transmitting ticks can be attacked, it is necessary to understand their live cycles and their relationships with the Babesia parasites

in Colombia. It is important to know which ticks are transmitting babesiosis in Colombia because if the principal vector should be a one-host tick, the possibility of eradicating the tick exists. Such a program would be very beneficial for the control of babesiosis in Colombia.

Since Boophilus microplus is probably the principal vector of Babesia in Colombia, it is also necessary to study the life cycle of the Babesia parasites in ticks. This is necessary in order to understand and develop the proper measures for the control and eradication of babesiosis in cattle.

Objectives:

To study the life cycle of Boophilus microplus, its distribution and population in various environmental conditions in Colombia. To determine if Boophilus microplus transmits only Babesia bigemina and Babesia argentina. To study the possibility of other ticks (Anocentor nitens) transmitting Babesia bigemina and Babesia argentina in cattle. To determine which stage of the life cycle of the tick is most efficient in transmitting babesiosis.

Material and Methods

Studies in vivo. Intact and splenectomized animals 2-6 months of age will be used for this experiments. The animals will be exposed to ticks known to be infected with Babesia bigemina or Babesia argentina. The engorged females will be kept under optimum

conditions, temperature and humidity to produce eggs. Larvae, nymphs and adults will be used for transmitting Babesia bigemina and Babesia argentina. Responses to tick-borne challenge will be determined by percent of parasitemia (P), packed cell volume (PCV), antibody titers (AT) and mortality (M).

Studies in vitro: Smears from the various developmental stages of ticks will be made and stained with Giemsa, Acridine Orange and fluorescent antibody in order to detect developmental stages of Babesia parasites. For these purposes a light and ultraviolet microscope will be used, however, electron microscope studies could be substituted and would be much more significant. In cooperation with the Universidad del Valle in Cali and the Instituto Nacional de Cancerología in Bogotá, this part of the experiment (electron microscopy) could be performed.

6. To study the pathogenesis of a triple infection with Babesia bigemina, Anaplasma marginale and Trypanosoma vivax in intact calves, utilizing serological, clinical, clinical pathological, histopathological and immuno-fluorescent methods.
7. To study the Babesia complement fixation antigen with column chromatography immuno-diffussion and immuno-electrophoresis.
8. To study the toxic effects of the Burroughs-Wellcome drug 4A65 in cattle.
9. To study the prevalence of babesiosis in Colombia.

IV TRYPANOSOMIASIS

A. Trypanosomiasis Projects in Progress

1. A Sequential Study of the Pathogenesis of *Trypanosoma vivax* in Experimentally Infected Calves, Utilizing Clinical, Pathological, Histopathological and Immuno-fluorescent Techniques.

Objectives :

To study sequentially the pathogenesis of a recent bovine isolate of *Trypanosoma vivax* in calves utilizing clinical, pathological, histopathological and immunofluorescent techniques.

Literature:

Trypanosomes that affect cattle have characteristics that place them in the genus *Trypanosoma* (20). Hoare described this form of flagellate as having a post nuclear kinetoplast, with the flagellum arising near it and emerging from the side of the body to run along a lengthy undulating membrane. Cyclical transmission of the parasites is limited to *Glossina spp*. which are the tsetse flies (23). Maturation to the infective stage occurs in the salivary glands of tsetse flies, therefore these trypanosomes are known as salivarian trypanosomes.

Kubes, in 1944, stated that T. *vivax* has been spreading through certain countries and adjacent islands in Central America since the second decade of the present century (22). Work by Kubes in Venezuela indicated that *T. vivax* existed

in that area, based on morphological data. Comparisons of the organisms found with smears of T. vivax from original African work were in agreement.

Wells, et. al. in 1969 found numerous instances of cattle of all ages infected with T. vivax in Colombia, South America (33). Current efforts to determine a vector are in progress, as there are no known tsetse flies in the New World. The cattle were also commonly infected with Babesis spp. and Anaplasma marginale (33).

The importance of bovine trypanosomiasis in South America is unknown, and it would be beneficial to study the epidemiological and pathological relationships of trypanosomiasis to other hemoparasites. In Colombia, in contrast to Africa, there have been no reports of Trypanosoma congolense in cattle, providing an opportunity to study T. vivax in cattle without complications from other trypanosomal species.

Trypanosomiasis in Africa limits animal production. Lumsden quoted Hornby as saying that trypanosomiasis denied large areas to all livestock production except poultry (23). Use of the affected area would support an estimated 125,000,000 head of cattle, which would be 11,000,000 head more than the total number of cattle in Africa in 1962.

The World Health Organization Expert Committee on Immunology and Parasitic Disease has indicated that there is a vital need to secure control of trypanosomiasis in animals and man and that progess has been slow so far in vector (Glossina spp.) eradication and chemotherapeutic approaches (36). Lumsden throught that consideration and investigation

of the pathological processes concerned in trypanosome infections may be significantly informative (23).

The pathogenesis of trypanosomiasis in animals has been described by Jubb and Kennedy as having escaped better than cursory attention, and regrettably little can be said of it except for a few observations on Chagas' disease in dogs (21).

Bovine trypanosomiasis was studied in detail near the turn of the century in Africa by Bruce (23). The disease was associated with a chronic cachectic condition rather than with a fulminant parasitemia, with minimal numbers of parasites observable. The term "Nagana",meaning "weakness", was used by Bruce to include T. brucei infections in horses, cattle, donkeys and dogs (but excluded swine). It is now known that an early shipment of biological material to Europe resulted in the failure to recognize that other Trypanosoma spp. were present. For this reason early workers thought that only T. brucei was involved in domestic animal infections.

In acute infections with a high parasitemia, it is reasonable to accept whatever Trypanosoma species that is present as the cause of death. However, in many cases of trypanosomiasis the course of the disease is lengthy, and usually few trypanosomes are visible in blood smears in the latter stages of disease. It is therefore risky to attribute the initiation of the disease process in all cases to the particular species observed in peripheral blood in the chronic stages. Bearing this in mind, T. vivax is the organism most often observed in chronic bovine cases of trypanosomiasis in West Africa, and Trypanosoma congolense ranks second, with

the order being reversed in East Africa (23). However, Fiennes, working in East Africa, stated that T congolense was associated with an acute or chronic type of disease usually ending in death of the bovine host whereas T. vivax often caused either a chronic or benign disease with a high recovery rate, and only exceptionally caused peracute or acute disease (10).

Because of the lack of information on the pathogenesis of T. vivax, the behavior of other Trypanosoma spp. has been reviewed. Gordon, et.al. have given a good understanding of the immediate fate of Trypanosoma rhodesiense upon being deposited in certain vertebrate hosts by the carrier insect (17,18). There was a rapid invasion of the blood by the trypanosomes. In the rabbit, but not in the rat or the guines pig, there was local multiplication at the site of the tsetse fly bite, resulting in a chancre formation similar to that occurring in man, and the trypanosomes produced were indistinguishable from those forms found in the blood stream. Chancre formation was thought to be a more local complication that accompanied invasion of the blood in some hosts but not in others.

The mechanisms of the pathogenesis of T. congolense in cattle have been studied in Africa by Fiennes et. al. In an early trial it was noted that young animals usually survived, whereas adults usually died (8). An endocrine factor was postulated to explain certain metabolic changes observed. No observations were made regarding T. vivax.

In an subsequent examination of histologic sections, Fiennes found multiple thromboses of the smaller blood vessels occurring

throughout the parenchymous organs of the bovine body in T. congolense disease, caused by multiple infarctions with resulting degeneration and replacement fibrosis of the parenchyma (9). The tunica adventitis became hyperplastic with a loss of elasticity, and the endothelial cells underwent metaplasia and passed into the general circulation during the stages of maximum macrophage mobiliztion. Favorable conditions existed for the development of thromboses.

Evidence that some trypanosome species in mammals are influenced by temperature is suggested by the following three reports, and a similar effect may occur with T vivax . A report from India supported the theory that a main habitat of Trypanosoma evansi in the vertebrate host was in the capillaries of the skin (1). Several cattle developed skin lesions that contained trypanosome-like organisms, but the chronic constitutional syndrome which was expected in Indian cattle with trypanosomiasis was not observed. Fiennes found T.congolense infections of the skin of cattle, and noted the rapid removal of the parasites once the main circulation of the body was encountered (9). He stated that serum antibody levels and in vivo temperatures above 38°C. were prime factors in the defense mechanism. Work by Marinkelle, using highly pathogenic Trypanosoma cruzi in mice, indicated that a high (36°C) environmental temperature protected mice for months, compared to 27°C. in the control group (24). It is apparent that the anatomical locations of pathogenic trypanosomes in mammalian hosts are not well understood. Recently developed immunofluorescent techniques offer a detection system not formerly used

in the study of the pathogenesis of trypanosomiasis.

Seed and Gam infected rabbits with Trypanosoma gambiense which developed skin lesions containing high concentrations of trypanosomes (30). The authors' working hypothesis stated that as agglutination and protective antibodies developed in the host, the trypanosomes were agglutinated and lysed. It was believed the dying organisms either released a toxin or certain antigens which triggered a hypersensitive reaction Either way the end results would be the same, namely tissue destruction and release of somatic cell antigen(s) not recognized by the rabbit host as self. Development of auto-antibodies would accelerate cell destruction, metabolic disorders and finally death.

Goodwin and Hook found that the subcutaneous introduction of T. brucei in mouse blood into rabbits resulted in a chronic angitis (16). Venous drainage of muscles and viscera was congested, tissues were edematous and vessel walls were fragile and surrounded by granulamatous tissue. Phagocytes were seen lining venules and capillaries, suggesting a long standing vascular lesion. Arterial constriction in the ears and cremaster muscles, mediated through the autonomic nervous system, produced anoxia and then necrosis. The vascular damage may have resulted from pharmacologically active substances from tissues due to and allergic reaction. The description of this lesion somewhat resembled the vascular lesions of the bovine species seen by Fiennes (9).

Goodwin and Hook quote Cunningham, Van Hoeve and Grainge, (1965), as saying that a series of variant strains of trypanosomes and their consequent antibodies appeared in cattle infected with T. brucei (16). Evidently, the trypanosomes as originally introduced into the host caused antibody formation, which in turn tended to eliminate the organisms. However, the trypanosomes apparently have the peculiar property of undergoing sufficient alteration in antigenic identity that survival agains the recently formed antibody was possible. In due time a second antibody titer developed, and repetitive cycles occurred. McNeillage et. al. reported with T. brucei that infectivity and pathogenicity appeared to be related phenomena and both varied according to the antigenic type involved (25). Weitz, working with T. brucei, stated that the number of variant trypanosome strains and resultant antibodies formed was practically endless (32).

Gray infected goats and rabbits, and studied the antigenic character of strains of T brucei transmitted by Glossina morsitans and G. palpalis. There was no change in the antigenic character of the trypanosome strain until the seventh day of the infection, after which new antigens developed at 2 or 3 day intervals until death. The same result occurred upon using different species of flies (19).

According to Boreham, cattle chronically infected with T. brucei displayed increased kinin activity 2 or 3 days after peaks in parasitemia occurred, and the precursor of kinin decreased in amount in the blood at the same time (4). The kinin release was thought

to result from an antigen-antibody reaction due to either causing local tissue damage, with subsequent release of kinins, or to activating the plasma kinin system, and hence, liberating kinin which caused permeability changes in the vascular endothelium. In either case a vicious cycle was thought to develop, with subsequent release of more kinins. The common antibody, not the variant antibody, was considered the antibody involved. The author quoted Cunningham and Van Hoeve who suggested that precipitinogens are continually being released throughout trypanosome infections, and that these are important in the development of the pathological changes which occur in trypanosomiasis.

Lumsden believed immunological mechanisms operating in protozoal infections, including trypanosomiasis, were similar to those operating agains bacteria and viruses, and there was no need to invoke special concepts (23). There now are, however, terms associated with certain protozoal immunological conditions (6). "Premunition," or "infection immunity" describes a condition when a protozoan parasite lives in a host in a latent state with the host being in a state of immunity or increased resistance to further infection because of the latent infection. Lumsden has stated that it will usually be impossible to separate premunition from sterile immunity, the latter term inferring that the host continues immune to further infection despite the complete elimination of the infecting agent.

Fiennes chose to use a new term, "cryptic infection", to describe the stage of premunition in chronic trypanosomiasis (11). The stage occurred in T. congolense infections seen to terminate in death. A mouse protection test was employed by Fiennes in

determining the presence of T. congolense in the cattle. Mice infected with T. congolense were exsanguinated at the height of the parasitemia. The citrated mouse blood was diluted and incubated with cattle (test) sera, and then inoculated into normal mice. Absence of a parasitemia was considered evidence that the sera contained antibodies to T. congolense, and that the cattle tested still harbored T. congolense. In some cases no evidence of antibodies was demonstrable.

Clinical pathological observations in bovine trypanosomiasis involving T. vivax included decreased hemoglobin values and erythrocytic counts, disappearance of eosinophils, neutropenia and periodical monocytosis associated with a reduction of circulating trypanosomes (11). Immature granulocytes decreased in number. T. vivax infection was capable of initiating the disease process in the absence of a demonstrable parasitemia suggesting the pathogenicity of trypanosomes was not related to parasitemic levels. The anemia of primary and secondary trypanosomiasis was similar, and in the latter stage, the chronic signs seemed to arise from an unknown tissue focus, whereas the acute signs of the primary disease were associated with an active parasitemia. The pathogenesis of primary and secondary trypanosomiasis was indicated to be different, yet some of the signs were similar. If the primary demonstrable parasitemic stage of trypanosomiasis was inhibited by chemotherapy, the secondary stage would still develop.

Chronic foci of both T. congolense and T. vivax in bovine cardiac tissue were found by Fiennes (12). Affected cattle had identical lesions and were from a group exposed to tsetse flies

followed by treatment with trypanosomicidal chemicals. Most of the organisms, which were thought to be mostly T. congolense with some T. vivax, were degenerated or lysed, but some normal forms were also present. Such a method of examination appears unsuited to precise identification of trypanosomes, and points out the potential of the immunofluorescent antibody technique. Fiennes found the organisms were usually in capillaries, although the location of some were undetermined, and the affected capillaries were always dilated. Myocardial necrosis varied in degree, but often was very severe. Some necrotic lesions appeared to be secondary to atrophy, due to either capillary parasitic emboli or pressure from dilated capillaries. It was suggested that the cardiac lesions may be the cause of the pathological condition in the secondary phase of bovine trypanosomiasis.

Gross lesions were described on treated and control cattle exposed to T. vivax (13). In the control group of cattle, subepicardial and subendocardial hemorrhages suggested a more acute cardiac involvement than in treated cattle, and renal medullary hemorrhage was more intense. Splenomegaly, edematous lymph noses and a decreased relative quantity of the red marrow of the long bones were common in both groups. General hyperplasia of hemal lymph nodes was striking in treated cattle. It was throught that treated cattle constantly exposed to infection developed a form of modified acute trypanosomiasis which did not correspond to the chronic, post-critical form.

In summarizing the early work of other investigators in trypanosomiasis, Fiennes commented on several theories (10). Direct intervention by the parasites could not be ascribed to the

general tissue necrosis of the several affected vital organs, as detailed examinations had never revealed any parasites. Developmental intracellular forms of the trypanosomes have been proven only with T. cruzi. Anemia was always severe in bovine trypanosomiasis, but did not vary in intensity between acute and chronic forms, and therefore, did not seem to be related to the progression of the tissue lesions. The theory of the development of tissue lesions caused by the introxication by dead trypanosomes has not been proven. Anaphylactic shock has been considered a possible explanation in peracute deaths due to trypanosomiasis. Other forms of excessive host reaction have been postulated, such as the periodic circulation of dead trypanosome antigen. An antigen-antibody union was thought to occur with injury to the somatic cells.

More recently the antigen-antibody tissue cell destruction theory has been discussed by Zuckerman (37). She pointed out that among the trypanosomiasis, the strogest case for autoimmunity can be made for those disease agents which include an intracellular phase in their life cycle. Four speculative models to adapt the various evidence for autoimmunication to protozoa are given. Model 1. The infectious agent might share an antigen or antigens with the host cell. Antiparasitic antibody might then combine with the heterogenetic antigen in the host cell. Model. 2. The target host cell might be coated with parasitic antigen, which could then unite at this site with antiparasitic antibody, with such a union injuring the host cell. Model 3. The target host cell might be directly coated with

antiparasitic antibody and be sensitized. Sensitized cells are more vulnerable than normal cells to certain mechanisms of host defense. Model 4. The target host cell might be so modified by an infectious agent and/or its products, or by a drug used in treating the infectious agent, as to become autoantigenic.

Adhesion of thrombocytes and leucocytes, and less commonly of erythrocytes, has been reported in trypanosomiasis of rodents, Equidae and primates (37). The factor responsible is commonly termed "adhesin," and is considered to be classical, nonprotective, antiparasitic antibody. It is not related to autoimmunity. The phenomena of erythrophagocytosis, autoglutination and augmented sedimentation rates are less completely understood. It is equivocal whether an antierythrocytic factor circulates simultaneously with antiparasitic adhesin to bring about these phenomena.

Zuckerman stated that protozoan diseases in which it was suspected that autoimmunity had arisen were all diseases of the blood and tissues where the organism had had intimate contact with cells and often were intracellular in nature (37). However, no intracellular forms of T. vivax have been demonstrated as has been done with T. cruzi, which develop in a series of mesenchymal cells, preferentially in cardiac muscle. A greatly diminished level of parasitemia was observed in blood smears in the chronic pahse of T. congolense infections in cattle (10). In this phase it was believed that the location of residual concentrations of parasites may have been intracellular

either in macrophages or other host cells.

The processing of tissues in chemicals is known to alter the host cell membrane, permitting entry of the specific immunofluorescent tagged antibody into the cell, therefore fluorescence and visual detection of the trypanosomes or their remains is feasible in the chronic stage of trypanosomiasis.

In summary, bovine trypanosomiasis is considered a serious obstacle to the increasing world demand for animal protein. T. congolense and T. vivax in Africa are well documented as inhibiting cattle production. In Central America and South America, T. vivax is known to be present in clinically ill cattle that are free of T. congolense, providing an opportunity to study the effect of T. vivax alone in cattle (22,33).

The recently developed immunofluorescent techniques seem particularly suited to detection of the locations of trypanosomes as they may exist in the various phases of the disease progress. Such a technique has not previously been reported in the literature in the study of the pathogenesis of bovine trypanosomiasis, and would have potential as a diagnostic method in this disease.

Material and Methods:

Fifteen intact, male Holstein calves of 4 to 6 months age were used in the experiment. One animal served as an experimental control. Prior to experimental infections, pooled aliquots of blood from each of the 14 experimental calves were examined for the presence of hemoparasites. Blood was inoculated into a susceptible splenectomized calf, cultured on enriched blood agar (34),

inoculated into a minimum of two laboratory mice at the rate of 1.0 cc. intraperitoneally per mouse, and observed in wet mounts in order to ascertain if any of the animals were already infected with trypanosomes or other hemoparasites. The calves used, originated from the Sabana of Bogotá, and were subjected from birth to weekly blood examinations for trypanosomiasis and serological examinations for anaplasmosis and babesiosis with consistently negative results.

For one week prior to infection the calves were examined at 2 day intervals to establish base line values of blood glucose concentrations (26), glucose-6- PO_4 dehydrogenase levels (3), sorbitol dehydrogenase levels (14), serum glutamic oxalacetic transaminase concentrations, total serum proteins (2,7) bilirubin concentrations (15), total and differential leucocyte counts and serum protein electrophoresis (5). A preinoculation bone narrow biopsy was taken from each calf. Body temperatures were recorded twice daily.

Each of 14 calves were infected on the same day with jugular blood that was examined with a hemacytometer and diluted so that each calf will receive approximately the same number of organisms. Quantities of the same blood were frozen also, insuring a stabilate (as defined by Lumsden) of trypanosomes for future work (23). The jugular blood was from a calf with an ascending parasitemia of

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* Hycel, Cyanmethemoglobin technique, Houston 2, Texas.

T. Vivax (Neiva, Colombia, S.A. isolate, 1969). The isolate was obtained from a cow showing clinical signs of disease in September, 1969, at Neiva, Colombia, and determined to be T. vivax by morphological examination, determination of non-infectivity following injection into laboratory mice and by culturing (33,34). The isolate was passed once in sheep to eliminate survival of any Babesia spp. It was then passed in a calf which was treated chemotherapeutically* to eliminate any Anaplasma marginale. Serum from the calf was subjected at intervals to complement fixation tests for evidence of anaplasmosis and babesiosis with negative results.

The calves were euthanatized singularly and at random at post-inoculation times of: 12 hours, 1, 2, 3, 5, 8, 12, 17, 23, 30, 38, 47 and 57 days. This logarithmically patterned experimental design permited a maximum number of observations early in the course of the disease process, including the time immediately after inoculation and before a parasitemia becomes evident. The period of 57 days assured adequate coverage of the chronic phase. The control animal was the last calf to be euthanatized. At each scheduled time of necropsy, 3 calves were selected at random, and bone narrow biopsies were taken, after which one calf was euthanatized.

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* Burroughs-Wellcome Co., Tuckahoe, N.Y., B-W Drug No. 356-C-61, and Chas. Pfizer Co., Inc., N.Y., N.Y., Oxytetracycline HCL.

Selection of each group of three calves was made from animals not having experienced post-infection biopsies, until each individual calf had received one post-infection biopsy, after which the process was repeated. At the scheduled time of necropsy, blood was collected from all of the calves remaining in the experiment for determination of the parameters listed above. Routine procedures were standardized as far as was practical recognizing that numbers of T. congolense fluctuate widely in peripheral blood smears within a 24 hour cycle (10)

A minimum of 3 sets of tissues were collected, one being preserved in formalin for staining with hematoxylin and eosin techniques. Two sets were frozen and stored in separated freezers for study by indirect immunofluorescent antibody techniques. Tissues were collected from any gross lesions. In addition, tissues from the following anatomical sites were taken: lip, tongue, dental pad, ear, nasal mucosa, mandibular lymph mode, semilunar ganglion, semitendinosus muscle, diaphragm, mediastinal lymph node, lung, heart, aorta, bone marrow, adrenal, kidney, urinary bladder, spleen, pancreas, rumen, reticulum, abomasum, duodenum, ileum, colon, liver, gall bladder, ovary, uterus, penis, testicles, cerebrum, cerebellum, medulla oblongata, chorioid plexus, pituitary, spinal cord, and bone. All routine samples of a given organ were taken from similar locations within the organ.

Specific hyperimmune serum for the Neiva, Colombia isolate of T. vivax was prepared by inoculating a susceptible calf. Rabbits were inoculated with bovine gamma globulin to produce anti-bovine gamma globulin serum. The fractionation of the serum globulins

and their labelling with fluorescein isothiocynate were removed by column chromatography (28). The specificity of the immunofluorescent labelling by the fluorescent tagged antibody was determined by the use of infected and uninfected tissues and by the inhibition of immunofluorescence by prior blocking of combining sites with untagged specific antibody.

The sequential study of the pathogenesis of bovine trypanosomiasis was made by utilizing clinical, clinical pathological, histopathological and immunofluorescent data obtained during the sampling and clinical examinations. An analysis of data concerning morphological lesions as they related to sequential clinical responses, degrees of parasitemias, levels of body temperatures, packed cell volumens and other parameters was made. Any lesions found on routine histological examination was studied further using indirect immunofluorescent antibody techniques. Blood and tissue impression smears were observed by immunofluorescence and other smears were fixed with methyl alcohol and stained with the Giemsa stain (31).

In September, 1969, and isolate of Trypanosoma vivax previously isolated from the Valle del Cauca was passed from carrier sheep into a calf, with the intention of using the isolate in the pathogensis study. The blood from the calf was monotored for some time to establish whether other hemoparasitic infections were present. Later an opportunity was presented to obtain a strain of T. vivax (Neiva isolate) from a cow with clinical trypanosomiasis. The Neiva isolate was passed in sheep and later to a calf. The calf blood was monitored daily for sometime, however, just before the pathogenesis study was

scheduled to begin, this carrier calf died without warning. It was then necessary to start again with another carrier calf treated 10 days with tetracyclines to eliminate anaplasmosis, utilizing infective blood from the carrier sheep to eliminate babesiosis. At this writing the blood from the new carrier calf has been introduced into a splenectomized calf, preparatory to beginning the main study.

Initiation of the experiment was delayed by two factors. Attrition of the pool of calves alloted to the trial necessitated obtaining more animals which have now been monitored to establish freedom of hemoparasites. Due to the inflexible vacation periods of essential laboratory personnel, another delay occured. Initiation of the trial was started on the day of return of these personnel on January 19, 1970.

Immediately upon arrival for work at this laboratory, several auxiliary activities were established. Monitoring of a daily lecture class on the pathology of the liver has helped the comprehension of Spanish scientific terms. A weekly meeting of a seminar on microscopic pathology also is being attended. Effort is contributed to the weekly routine bleeding of all animals under the jurisdiction of the Texas group.

A field trip was taken in company with a recognized expert in trypanosomiasis resulting in the isolation of the Neiva strain of T. vivax. Considerable time at the laboratory was made available also to gain experience in trypanosomiasis research. The proposal for the pathogenesis study was thoroughly discussed and some modifications were made.

All animals of the Texas group coming to necropsy have been subjected to autopsy and microscopic studies performed as the cases indicated. In normal raised animals in the enviroment of Bogotá and not having been subjected to experimental infections, there is considerable evidence that respiratory problems can be significant.

As opportunities arise, contacts have been made with the personnel in several branch diagnostic laboratories, and with practicing veterinarians. One technique peculiar to the animal husbandry is the used of atropine in bovidae being shipped by air to prevent bloat while at high altitudes.

Assistance has been given to other researchers including help at two off-site locations: Girardot and Bugalagrande. Such expeditions are quite beneficial in absorbing cultural features as well as scientific data.

As time has permitted, efforts have been extended to talking with the personnel at the research laboratories and at the college of veterinary medicine at the nearby National University, for example concerning the Aftosa work being carried on, but also including sociological topics.

B. Trypanosomiasis Projected Projects

1..To establish a bank of worldwide trypanosomes stored in a solid CO_2 compartment.

2. To establish the use of the indirect fluorescent antibody

test as a routine method of diagnosis in Colombia.

3. To study the pathogenesis of a triple infection of T. vivax, B. bigemina and A. marginale in intact calves.
4. To study the pathogenesis of a double infection of T. vivax and A marginale in intact calves.
5. To adapt a Colombian isolate of T. vivax to rats without the presence of sheep serum.

V. TRAINING IN HEMOTROPIC DISEASES AND RELATED AREAS

A. Graduate Courses Taught

1. Special Pathology - 3 hours credit. The course consisted of 3 lectures weekly for 6 months. The subject matter for the course has consisted of the embryology, macroscopic anatomy, microscopic anatomy, ultrastructure, biochemical and metabolic mechanisms, clinical pathology, infectious, nutritional neoplastic, immunologic and toxic diseases of the liver of domestic animals, man and laboratory animals. Eight students, of three different nationalities, took the course as a required subject for their Master of Science degree in Veterinary Pathology offered by ICA and the National University.

2. Special Microbiology.

   It is anticipated that the personnel of the Texas Group will participate in the teaching of this course which is required in the Master of Science degree program in Veterinary Microbiology offered by ICA and the National University.

In particular, the Texas Group will be asked to teach special research techniques such as protein and antigen electrophoresis in paper cellulose acetate, gel and stareh-gel media, double gel-diffusion, immunoelectrophoresis, sephadex anion-cation exchange resin column chromatography, enzymology, colorimetry, complement fixation, hemagglutination, latexagglutination, precipitation and agglutination serology.

B. Short Courses

1. Program of Training for Personnel of the ICA Diagnostic Centers: Laboratory Diagnostic Techniques.

The personnel of the Texas Group gave illustrated lectures on the prevalence, etiology, symptomology, epizootiology, pathology, treatment and control of anaplasmosis, babesiosis and trypanosomiasis to a group of 30 veterinarians employed in various diagnostic centers of Colombia. The short course consisted of 6 hours lecture and a one hour lab.

2. Second Program of Training for Personnel of ICA Diagnostic Centers: Laboratory Diagnostic Techniques.

The personnel of the Texas Group participated in the same way as given above.

3. Short course on hemotropic diseases was given to 60 Veterinarians at the University of San Marcos, College of Veterinary Medicine in Lima, Peru, May 17 until May 27.

C. Seminars

Seminars pertaining to epizootiology etiology, symptomology, pathology, treatment and control have been given at L.I.M.V. to ICA professionals and the National University undergraduates of the faculty of Veterinary Medicine.

D. Conferences:

1. General Aspects of Hemotropic Diseases were presented to the faculty and undergraduates of the school of Veterinary Medicine at the University of Caldas in Manizales.

The conference consisted of 8 hours of lecture, and discussion related to current research of hemotropic diseases and future projects as related to the incidence, epizootiology, pathogenesis, treatment, diagnosis and control of anaplasmosis, babesiosis and trypanosomiasis.

Plans were made for collaborative investigation into the incidence and prevalence of anaplasmosis and babesiosis in Caldas during 1970.

2. Livestock Production Specialist Training Project of CIAT. A short conference or course was given to 13 trainees in the Livestock production program of CIAT. Basically, the conference consisted of 8 hours of demonstrated lectures pertaing to the pathology, pathogenesis, transmission, prevalence, etiology, control, diagnosis and treatment of

anaplasmosis, babesiosis and trypanosomiasis, followed by a discussion and a laboratory demonstration. Another lecture was presented on the pathology, diagnosis and treatment of poisonous plant, heavy metal, insecticide and miscellaneous toxicology.

E. Graduate Training and Research

1. The Texas Group personnel are presently serving as chairmans of 4 Master of Science degree thesis committees in veterinary pathology and microbiology in the ICA and National University programs. The personnel also serve as members of 6 other master's committees in veterinary pathology and microbiology.

The titles of some of the proposed theses include:

A Study of the Pathogenesin of Babesiosis in Intact Calves, Including Clinical, Clinical Pathological, Serological, Histopathological and Immunofluorescent Methods;

Exfoliative Cytology of the Estrous Cyble of Marez;

Comparison of the Morphology, Antigenicity and Cross-Immunity of Colombian Isolates of Babesia bigemina;

Alterations of Bovine Serum Proteins in Pure and Mixed Infections with Anaplasma marginale and Trypanosoma vivax.

The Texas Group Advisors are responsible for aiding the students in the proposed thesis problem and equipment, laboratory space, reagent and experimental animal procurement as well as giving technical advice.

F. Attendance and Participation in Scientific Meetings

1. American Veterinary Medical Association convention. A paper entitled "The Incidence of Bovine Babesiosis in Colombia, South America" was presented.

2. The Conference of Research Workers on Animal Disease was attended

3. Seventh Colombian National Congress of Veterinary Medicine and Animal Science

   Several papers were presented including: The Clinical Pathology of Bovine Babesiosis; Fine Structure of Babesia bigemina; Pathology of Equine Onchocerciasis; Neonatal Ovine Necrobacillosis; Bovine Dermatophilosis (Cutaneous Streptothricosis) in Colombia; Diagnosis of bovine Babesiosis in Thin and Thick Peripheral Blood Smears. The Neonatal Ovine Necrobacillosis papers was selected as being the best paper of the conference.

4. In 1970, it is anticipated that papers will be presented at the Sixth Panamerican Congress of Veterinary Medicine and Zootechnics, the American Veterinary Medical Association Convention and the Conference of Research Workers on Animal Disease.

5. American Veterinary Medical Association Convention (American Association of Veterinary Parasitologists) Las Vegas, Nevada, June 22-26, a paper entitled "Immune Response of Cattle Vaccinated Against Babesiosis in Colombia South America".

Section III

Institute of Tropical Veterinary Medicine

College of Veterinary Medicine

Texas A&M University

July, 1970

Studies on Hemotropic Diseases

Progress Report

Prepared by K. L. Kuttler

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Introduction

A major emphasis has been directed toward anaplasmosis, and its control in cattle, but in addition, studies have been conducted on *Babesia rodhaini*, *B. bigemina*, and *B. argentina* (Nuevo Laredo), *B. cervi*, and *Theileria cervi*. Studies with *B. cervi* and *T. cervi* in deer have been a continuation of work initiated before the Colombia project. Work at Nuevo Laredo has very limited objectives, over 50% presently completed, and will probably terminate within the next 6 to 8 months. *Babesia rodhaini* in mice represents an excellent laboratory model for basic studies, particularily with respect to immunologic properties of a *Babesia*. It is easy to work with, reasonable numbers of animals are possible, and the time lapse between exposure-infection and death is minimal making possible more rapid progress.

During the past year considerable time and effort has been expended acquiring necessary equipment and operating supplies to activate our research. We have now acquired most of the major-critical items needed, and are fairly well stocked with supply items. A major problem was solved when an agreement was made with the Texas Department of Correction for the regular purchase of weaned calves for experimental use. We have been receiving 6-8 calves each month since the implementation of the agreement in July, 1969.

Work on *B. cervi* and *T. cervi* in deer has been hampered by the shortage of experimental animals. This will probably continue to be a major drawback to work with these pathogens.

Considerable time has been spent acquiring and training technical help, for the laboratory, but we have been fortunate in finding well educated, competent staff which have learned quickly, and are now functioning efficiently.

Much of our work to date has been preliminary in nature, and is still incomplete, but studies have been and are in progress covering a number of areas which hopefully will provide basic information of use in the control of hemoparasites.

Anaplasmosis Treatment

Work previously reported (March, 1969) suggests that the α dithiosemicarbazone compound (designated 356C61, produced by Burroughs Wellcome Laboratories) is relatively more effective in the treatment of anaplasmosis than the tetracyclines. Serious problems were encountered when 356C61 was used in doses sufficient to eliminate infection. Toxic side effects were encountered which resulted in irreversible changes followed by death.

It has seemed that alterations of the number, and frequency of treatment might overcome these problems. For this reason a number of experiments have been designed to test various numbers of treatment at different times in order to test this hypothesis. In addition attempts to add agents to both slow and enhance absorptinn have been tried.

Treatment - Experiment #1:

Object: To determine the efficacy of 2 and 3 injections with in a weeks period, in removing the carrier status of intact calves.

Materials and Methods: 5 approximately 6 month old calves, all infected with anaplasmosis, as determined by a previous history and the complement-fixation test were used. Two calves 154 and 156 were injected Monday, Wednesday and Friday with 5 mg/kg 356C61 intravenously (I.v.). Calves 151 and 153 were injected on Monday and Friday with the same inocula. Calf 152 remained as an untreated control.

Four weeks after the last treatment, 10 ml of blood was withdrawn from each of the animals and checked for infectivity by inoculating splenectomized calves. Blood from 154 and 156 was inoculated into calf 197. Blood from 151 and 153 was inoculated into calf 190. Blood from 152 was inoculated into calf 196.

Results: Very little change in PCV was noted between the treated groups and the control. A general drop in CF titer was evident and most marked in calves 154 and 156 receiving the 3 injections.

The results of residual infectivity are given in Table 1. Calf 197 inoculated with blood from 154 and 156 (receiving the 3 treatments) remained non-infected. Calf 190 inoculated with blood from 151 and 153 (2 treatments) developed anaplasmosis 31 days after inoculation. Calf 196 inoculated with blood from calf 152 (non-treated controls) developed anaplasmosis 17 days after inoculation.

Calf 154 developed evidence of abdominal pain, restlessness, and went off feed. Severe bloating was not observed. These signs persisted about 5 days, then the animal returned to normal. No adverse reactions were observed in any other animals.

TABLE I

Effects of Treatment on Infectivity

Calves	Treatment Schedule	Effect of Treatment on CF Titers	Check for Infectivity	Incubation Time
154	Mon., Wed., Fri.	Lower	10 ml blood I.V.--197	No response
156	Mon., Wed., Fri.	Lower	10 ml blood I.V.--197	No response
151	Mon. - Fri.	No change	10 ml blood I.V.--190	31 days-Anaplasma
153	Mon. - Fri.	Lower	10 ml blood I.V.--190	Positive
152	No treatment	No change	10 ml blood I.V.--196	17 days-Anaplasma Positive

Conclusion: The treatment schedule on Monday, Wednesday, and Friday was apparently effective in eliminating anaplasmosis. Those receiving only 2 injections remained carriers. The increased incubation time in calf 190 as compared to calf 196 does reflect a drop in the level of infectivity in calves receiving 2 injections, when compared to the non-treated control (calf 152).

Treatment Experiment #2

This study was similar to experiment 1 except that a splenectomized calf, 119, with anaplasmosis, was treated 3 times, Monday-Wednesday-Friday with 356C61 at the 5 mg/kg level. This was done because some concern that the inate resistance of young intact calves might mask a persisting low level of infection even after treatment, which could become apparent later in life.

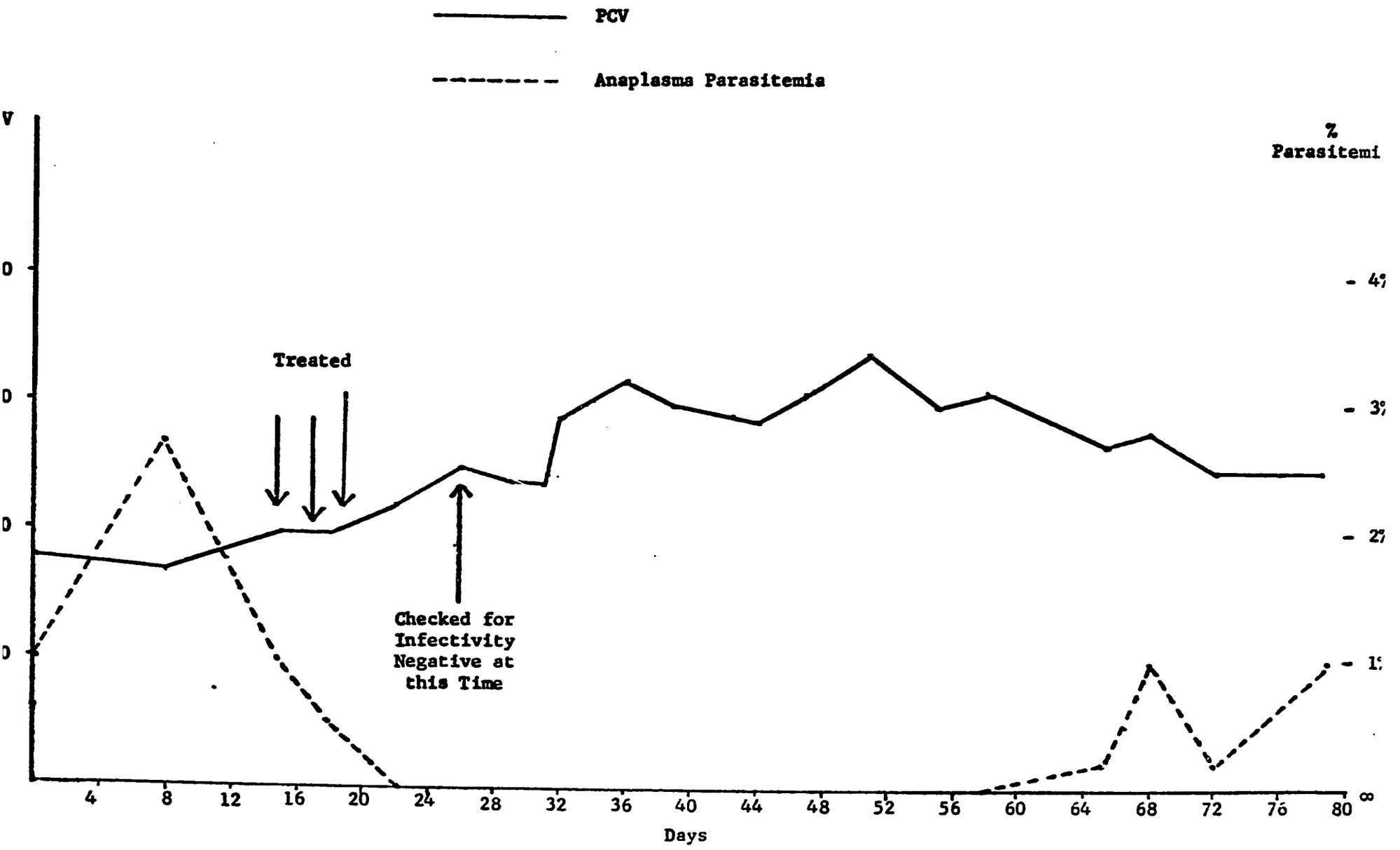
Results: Response to treatment is presented in figure 1. Calf 119 responded favorably to treatment. The PCV increased, the CF titer decreased, but did not drop below a 1:20 titer, and the parasitemia disappeared. Splenectomized calf 117 was injected with 20 ml whole blood I.V. from 119-12 days after the last treatment. No evidence of infection in 117 was observed 74 days following exposure. An anaplasmosis relapse in calf 119 was observed 49 days after the last treatment.

Conclusion: Infection could not be picked up 12 days after treatment, but was obviously present in the animal as evidenced by the relapse 49 days after treatment. Possibly a larger inoculum would have succeeded in picking up infection, but in any event if the organism was in the circulation it was present in exceedingly small numbers. These results do cast some doubt on experiment #1, since a relapse would not have been expected in an intact animal eventhough the infection was present.

Under normal conditions treatment will be important for intact animals only, but as a safety measure it would be good if we could remove the infection by treatment of splenectomized calves. If this can be done then there is little doubt that similar measures would be effective in intact animals.

FIGURE 1

PCV and Parasitemia of Calf 119, before and after Treatment with 356C61.



Treatment Experiment #3

Very often a single treatment will result in a prolonged period of no parasitemia, only to have a relapse finally occur. It was thought that there may be some merit in prolonging treatment, assuming that blood levels would remain reasonably high for a matter of days and possibly for as long as a week. It was hoped that intramuscular injections would result in somewhat slower absorption with more persistent blood levels.

Materials and Methods

A total of 7 splenectomized Holstein male calves--4-6 months of age were used for this trial. All calves had recovered spontaneously, without treatment from anaplasmosis. Calves 174, 176, and 180 were treated with 356C61 (group 1) at the rate of 5 mg/kg administered twice a week at 3 and 4 day intervals, for 2 weeks. A total of 4 treatments were given. All injections were intra muscular. Calves 181, 186, and 189 were treated in the same manner (group 2) except that the treatment interval was once a week for 3 weeks.

Calf 191 was not treated and served as a control for the 2 treatment groups. No infectivity checks were made but all animals were observed 3 months following treatment for evidence of relapse. Packed cell volumes, complement-fixation titers, and parasitemias were observed during the entire period of the test.

Results

Group response to treatment and no treatment is presented in figures 2,

3 and 4. Treatment in both groups was responsible for a temporary remission of evidence of infection, but in both groups infection reappeared. The appearance of a demonstrable parasitemia occurred most rapidly in animals 181, 186 and 189 receiving 3 treatments at weekly intervals. A diagnositc parasitemia of at least 1% was seen on an average of 41.7 days after the last treatment in animals receiving 4 treatments over a 2 week interval, and an average of 35 days for those receiving 3 treatments. A persistent, almost cyclical, parasitemia is noted in control calf 191. Treatment did interfere with this type of parasitemia, at least on a temporary basis.

Discussion and Conclusion:

The more frequent treatments appeared more valuable, being responsible for a longer period without parasitemia. In view of experiment #2 it seems possible at this point that the Anaplasma may persist outside the blood stream in a form not yet identified, and refractory to treatment, then reappears following treatment. The control animal 191 does show a cyclical reoccurrence of parasitemia and anemia which are closely associated.

Treatment Experiment #4

Concurrent with experiment 3 a treatment schedule of 5 mg/kg given I.M. and administered every 2 weeks for a series of 3 injections was attempted. This approach was prompted by the constant reoccurrence of parasitemia following treatment and in the 25-30 day cycle seen in non-treated controls. It was desirable to see if the period of no parasitemia could be lengthened by this approach.

FIGURE 2

Average PCV and Parasitemia of Calves 174, 176, and 180 before and after Treatment with 356C61.

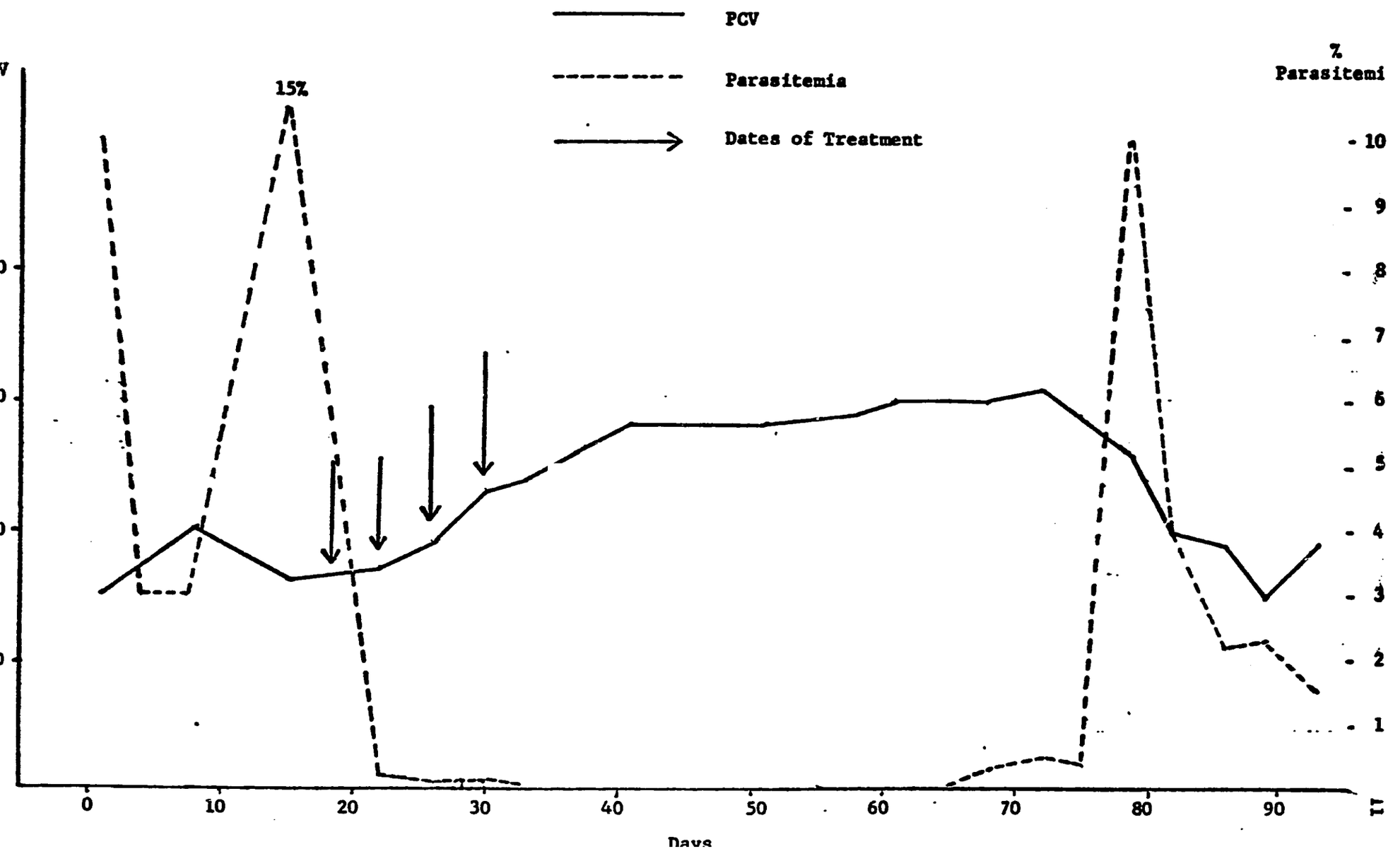


FIGURE 3

Average PCV and Parasitemia of Calves 181, 186, and 189 before and after Treatment with 356C61.

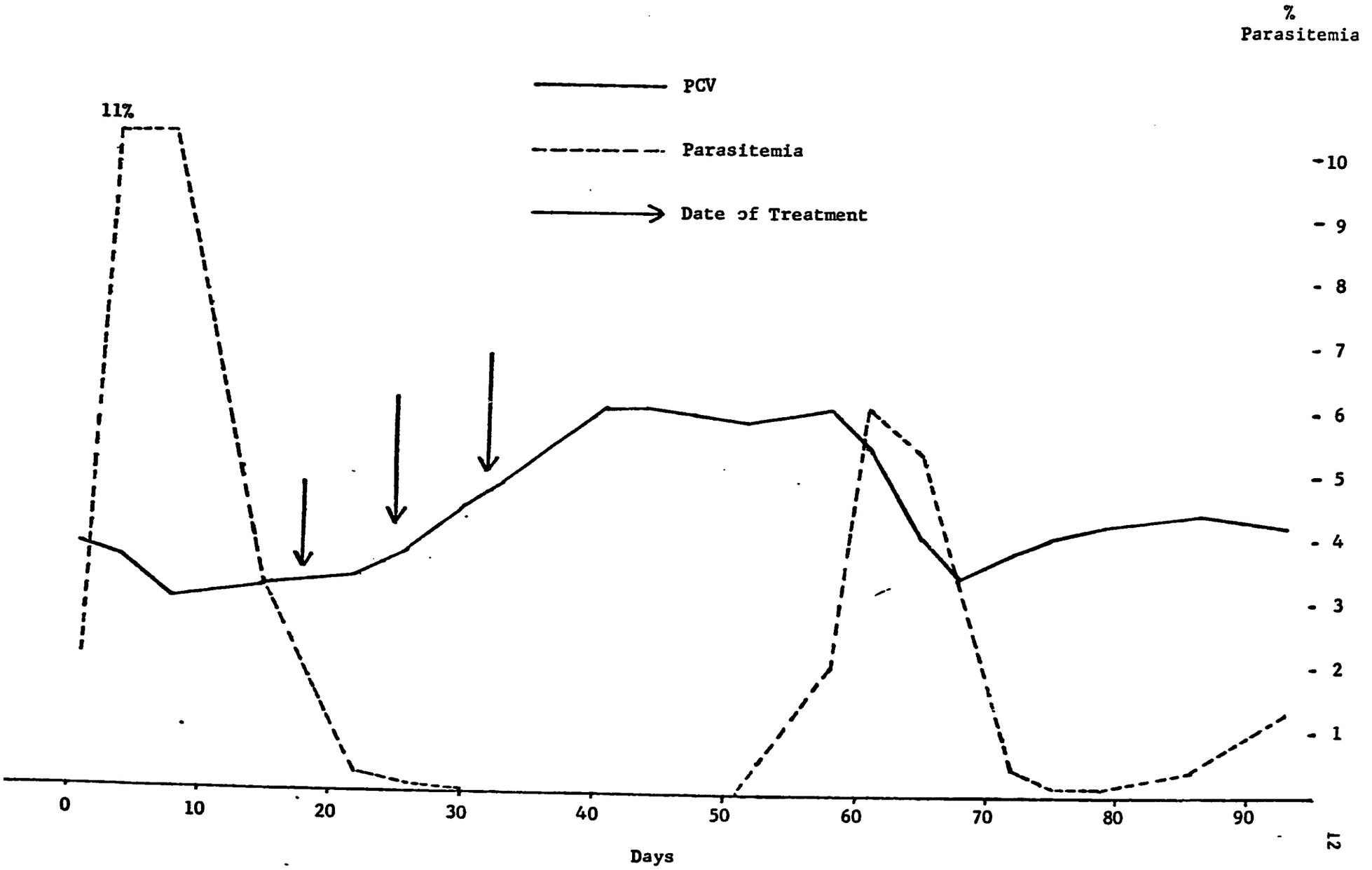
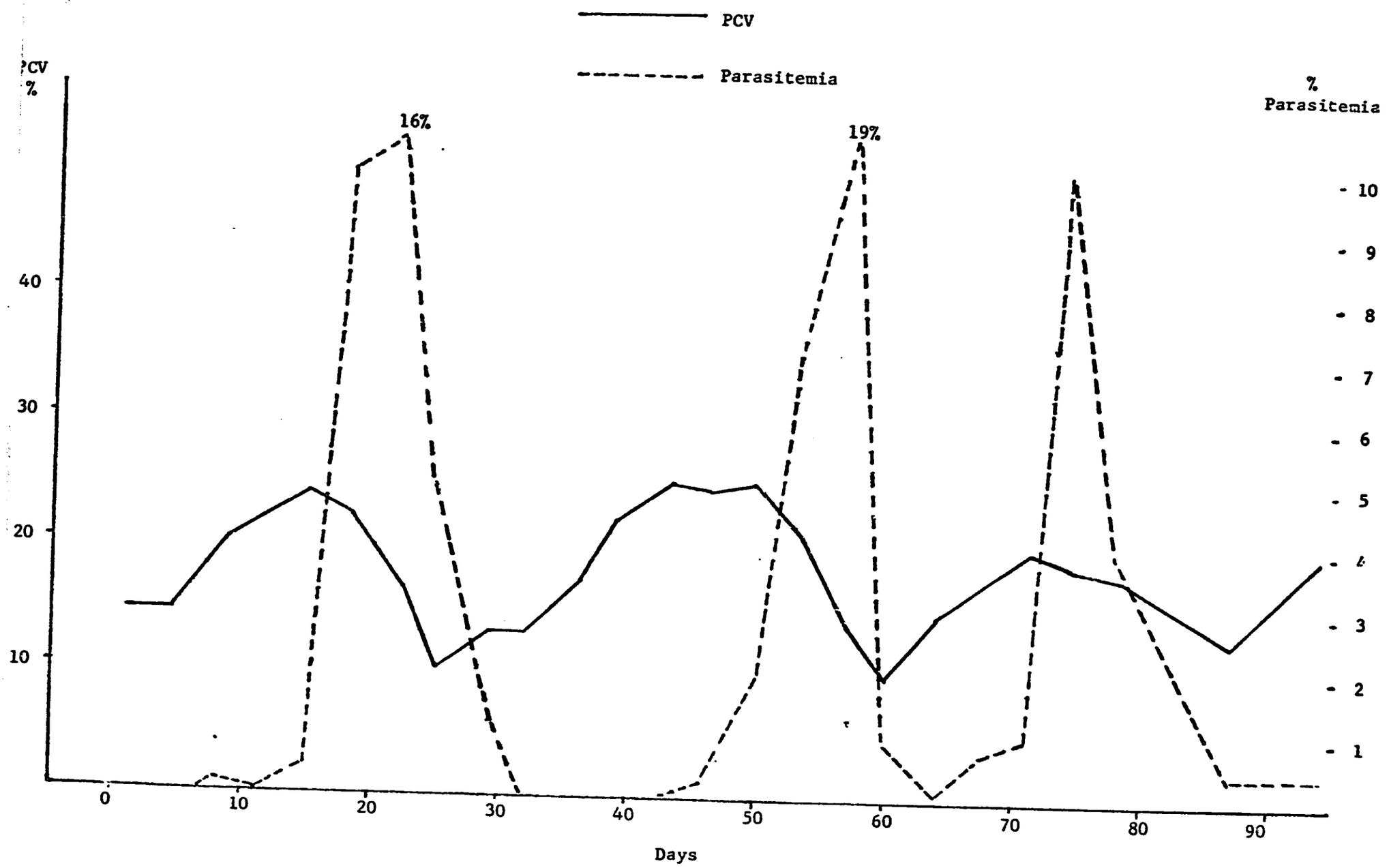


FIGURE 4

Splenectomized Calf 191 Untreated Control.



Materials and Methods

Splenectomized calf 117, showing typical persistent A. marginale parasitemias, was treated with 5 mg/kg 356C61 I.M. on day 1, 14, and 28. The usual observations were made following treatment.

Results

The effects of treatment in 117 are presented in figure 5. As previously seen an initial reduction in parasitemia was observed. Demonstrable parasitemia was not observed 8 days after the first treatment. This negative phase persisted until day 28 when the 3rd treatment was given. A low level parasitemia was apparent on day 28. The usual parasitemia regression did not follow the 3rd treatment as expected eventhough levels remained very low.

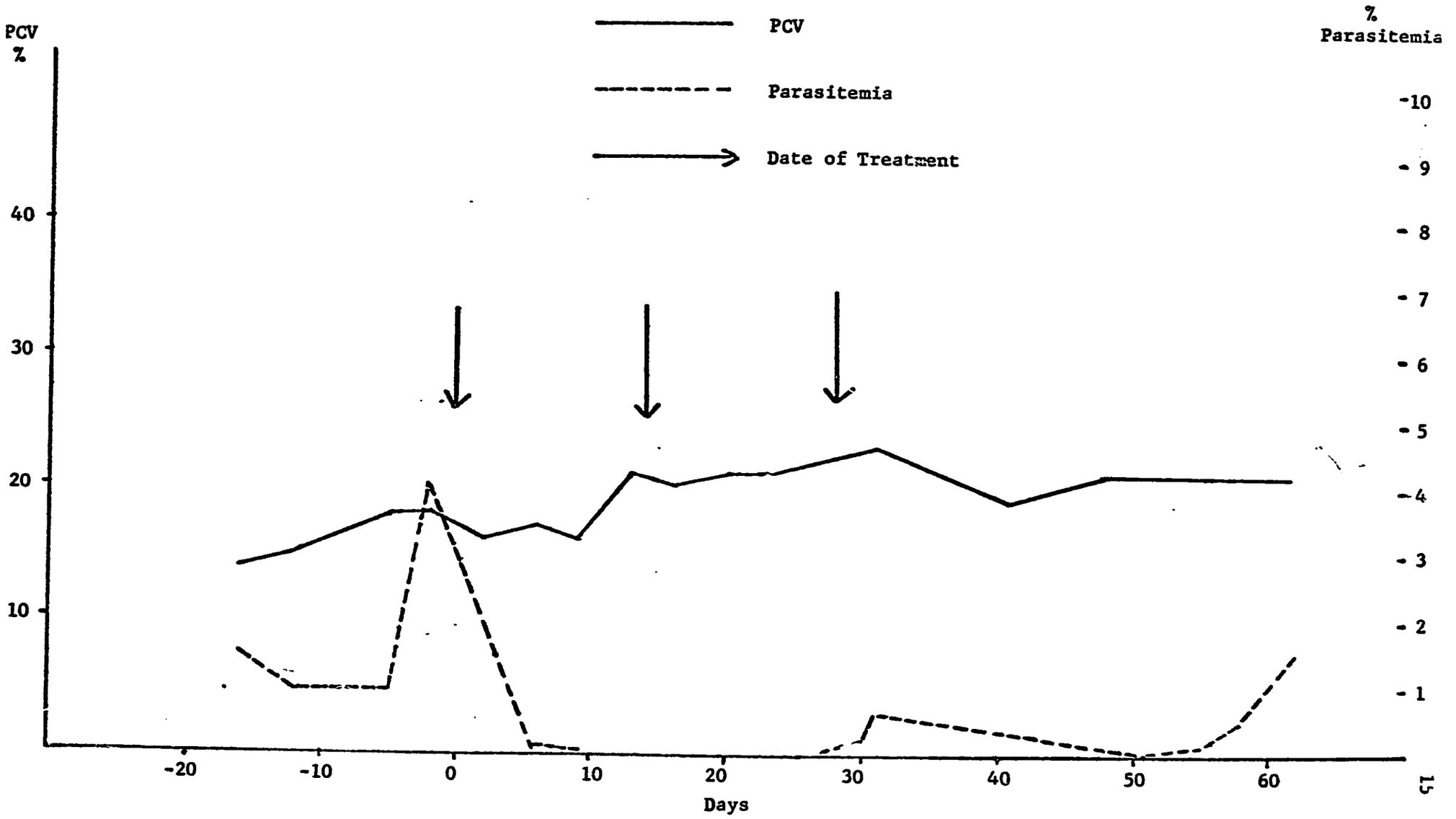
Conclusions

Experiment #4 substantiates the hypothesis that there is a phase in the life cycle of the Anaplasma that is refractory to treatment with 356C61. The regression of the Anaplasma organism appears to approach minimal levels very rapidly, but we have, to date, been unable to entirely eliminate the infection.

This resistance phase apparently can produce initial bodies, capable of invading the red cell. This occurs irrespective of the treatment being administered. Undoubtedly treatment is responsible for retarding the erythrocytic cycle, but the persistent low level parasitemia does suggest a source other than the erythrocytic phase that is not susceptible to treatment.

FIGURE 5

PCV and Parasitemia of Calf 117 before and after Treatment with 356C61.



Treatment experiment #5

Alterations in the route of inoculation, and the time interval using 356C61 have not resulted in a complete removal of infection. These trials using 2 splenectomized A. marginale infected calves have employed different carrier vehicles with the hope of increasing the effectiveness of the drug when administered at the same level.

Procedure

A. marginale carrier calf 190 was treated on day 1 with 5 mg/kg 356C61 administered I.M. The drug in an aqueous phase was mixed with mineral oil and adjuvant (Arlacel A) prior to injection. This material consisted of:

356C61 ----- 50%

Mineral oil - 40%

Arlacel A --- 10%

An emulsion was accomplished by sonication 5 minutes at 95 watts. A water bath was used to control temperature. It was hoped that absorption could be retarded, hence lengthening the negative blood phase.

A second splenectomized carrier calf 184, was treated on day 1 with 356C61 at the level of 5 mg/kg administered I.V. In this instance 10% DMSO was added to the basic drug in the hopes of increasing drug solubility and its penetration through cell membranes. By this approach a possibly more rapid action could be achieved, which might even be effective against the resistant phase of the organism.

Results

Results of treatment in animals 190 and 184 are present in figures 6 and 7. A demonstrable parasitemia disappears rapidly in both animals. Calf 190 receiving the adjuvant showed a reoccurring parasitemia 58 days after treatment and calf 184 a similar parasitemia 48 days after treatment. General improvement in PCV values accompanied the negative blood phase in both animals.

Conclusion

No real advantages to either the oil to retard absorption or the DMSO to increase absorption could be noted. No significant deviations from previous treatment trials could be detected.

Future treatment trials

1.) Alternate treatments with tetracycline and 356C61 will be attempted.

2.) Increased frequency of treatment using lower dose rate for longer periods of time will be attempted.

3.) A trial is presently underway similar to work done at Bogota in 1967 with the drug being administered at given intervals after treatment, with the object of determining the optimum time for treatment in establishing a pre-munizing infection.

FIGURE 6

Treatment of Splenectomized Calf 190 with 356C61 Mixed with Oils to Retard Absorption.

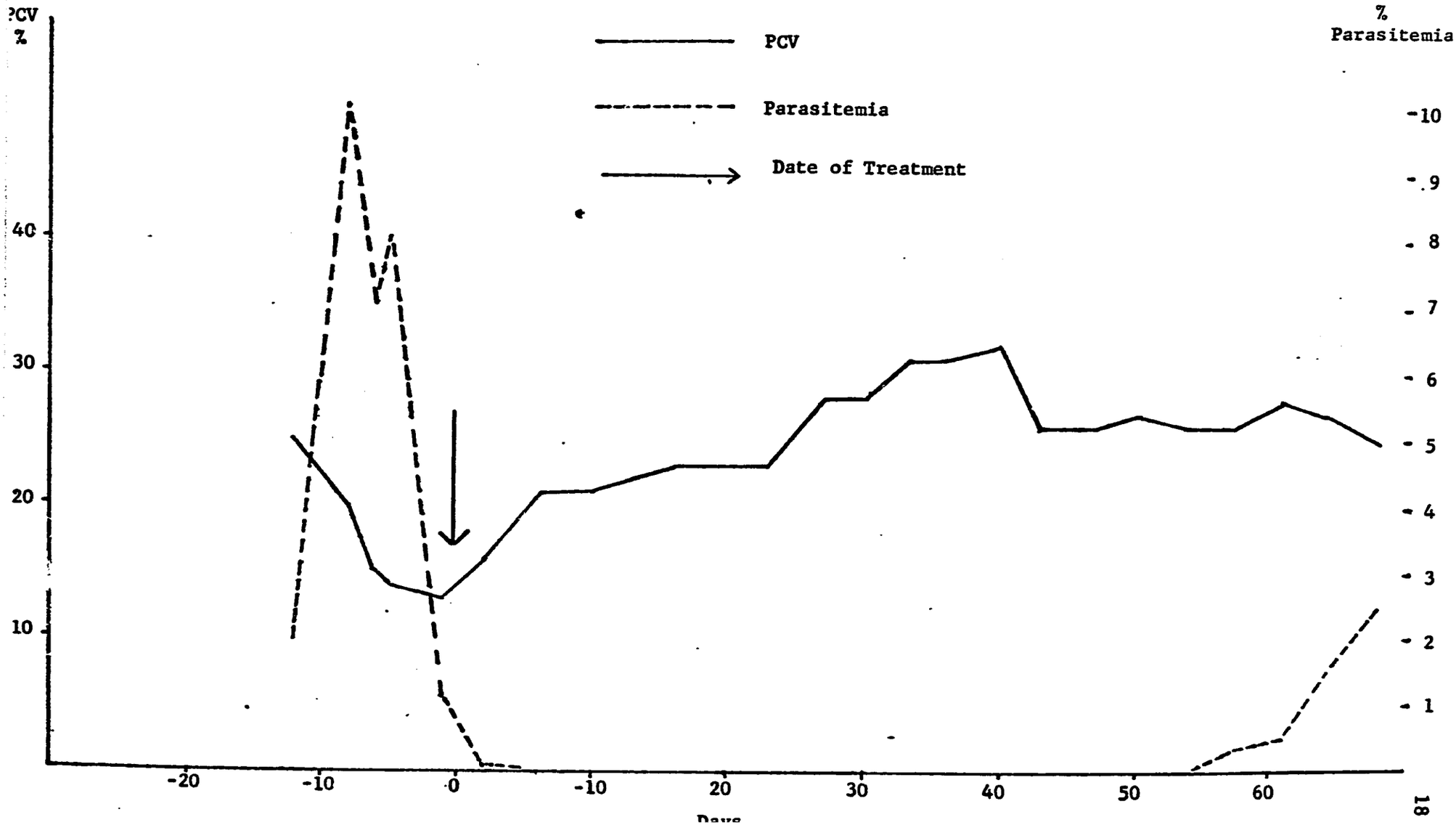
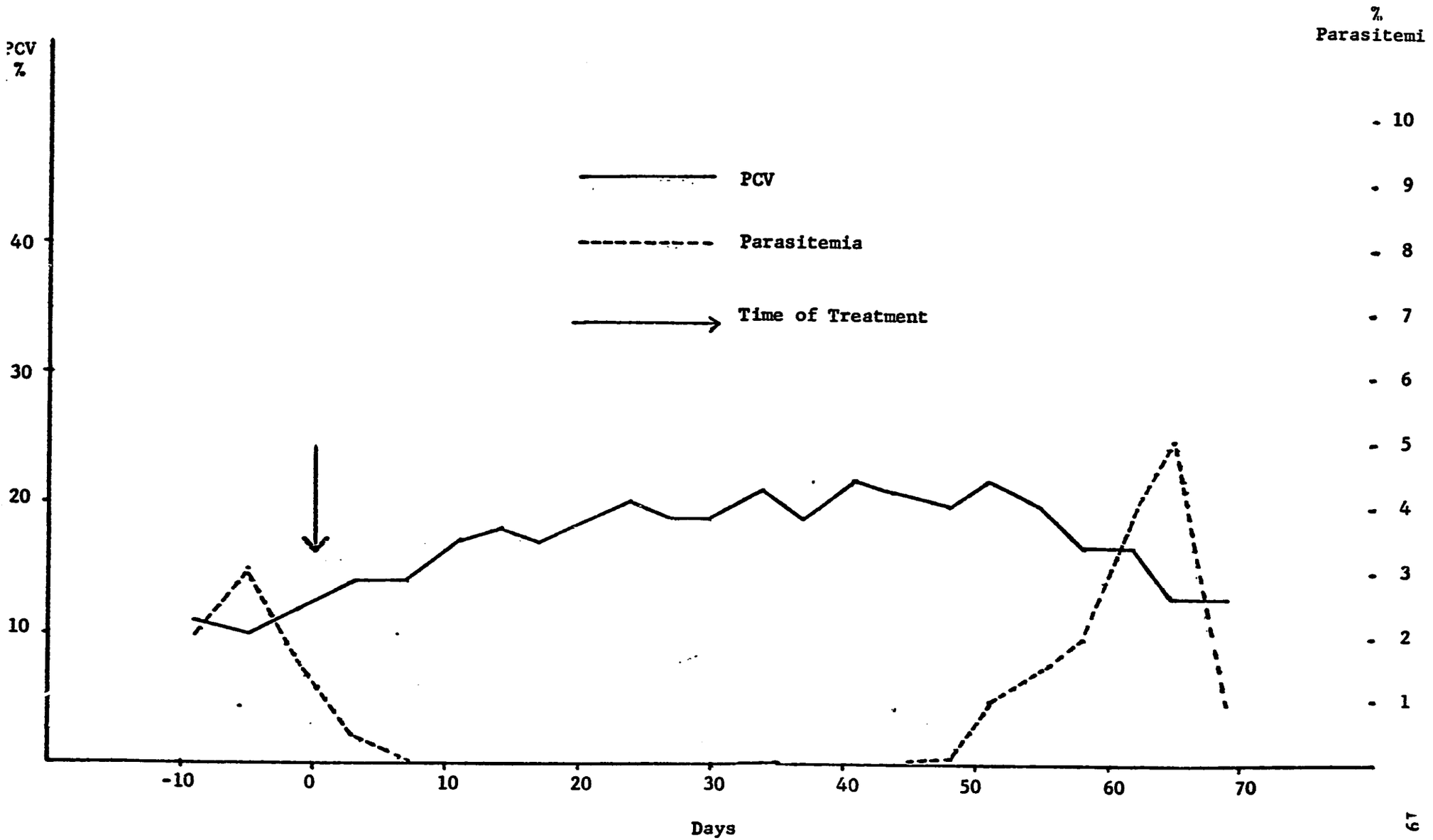


FIGURE 7

Treatment of Splenectomized Calf 184 with 356C61 Mixed with DMSO to Enhance Absorption.



Anaplasmosis Vaccine Studies

In recent months a problem has been encountered of iso-erythrolysis in new born calves from dams having been vaccinated with the killed anaplasmosis vaccine (Anaplaz). Evidence is being found to associate vaccination with this problem in calves, and although not proven at this time is sufficiently suggestive to require a more thorough look at anaplasmosis immunization. There are a number of changes that can be made in the vaccine to possibly circumvent this problem, and to actually improve the immunizing properties of a killed material. Work has been started with these objectives in mind.

A second possible factor which could become an outgrowth of this problem would be greater emphasis on live attenuated vaccines. One of the principle problems associated with this vaccine is the difficulty in handling the material in the field, and its susceptibility to storage. In addition this vaccine is mainly effective in those areas where anaplasmosis is due to a strain homologous to the vaccines origin. It is possible that additional strains may require attenuation, for inclusion with the present vaccine, thereby broadening the spectrum of protection.

These are some of the factors underlying our present work, which to date has been very preliminary in nature.

Vaccine Experiment #1

An Anaplasma vaccine prepared from adjuvants containing Mycobacterium butyricin (MB), in 1961, produced a marked resistance in splenectomized calves. Comparisons in 1965-66 indicated that the present commercially prepared vaccine was not sufficiently potent to protect splenectomized calves even when 2 injections of vaccine were given. The problems of local tissue response resulting from the MB adjuvant has discouraged the further use of this approach. With improved techniques for antigens concentration, it is possible that smaller inocula might be useful, which would produce less severe local reaction.

Method

Vaccine preparation: The basic material from which several antigen have been prepared were washed bovine erythrocytes showing a 50% parasitemia.

Lysis was accomplished by sonication. A sediment was collected by centrifugation at 35,000 XG for 40 minutes. This sediment was washed in cold distilled H_2O one time then resuspended in veronal buffer and labeled "RBC antigen". The supernate was acidified with 0.1N - HCl to a pH of 5.5. A second sediment was collected, washed, and labeled "Antigen 5.5". A third precipitate was collected when pH was further reduced to 4.5. This sediment was suspended in veronal buffer and labeled "Antigen 4.5". The aqueous antigens were emulsified with mineral oil containing 2 mg of MB per ml. The following show proportions of items used.

1.)	Anaplasma antigen	50%
2.)	Mineral oil (containing 2 mg MB/ml)	40%
3.)	Arlacel A (Emulsifying agent)	10%

Splenectomized calf 135 has been vaccinated with 2 ml of the RBC antigen

base I.M. on day 0 and day 28. Injections were made in the neck muscle. Splenectomized calf 136 has been vaccinated with 2 ml of the "Antigen 5.5" base in the same manner. Calf 137 has received a control vaccine containing only veronal buffer. All animals have been challenged with virulent A. marginale.

Results: No severe local tissue reactions were observed. CF titers have developed in both animals having received the Anaplasma antigens. Reaction to challenge has not yet occurred.

Comment: Serological response was not marked until after the 2nd injection. An earlier response is thought to be required in order to develop the desired immunity, but final evaluation will have to wait for the final results of challenge.

Vaccine Experiment #2

Basically this is a storage experiment which should assist in developing more satisfactory storage procedures for the attenuated or virulent organism.

Materials and Methods

This study involved the evaluation of DMSO as an additive to blood being frozen. Whole blood from C-152 showing a 0.5% parasitemia and a PCV of 18% was used. Blood was mixed with an equal part 6% DMSO in 0.85%. NaCl and frozen at -20°C for 72 hours then checked for infectivity by injecting the equivalent of 1 ml blood subcutaneously into intact calf 154. Blood was mixed with an equal part of 6% DMSO and frozen at -65°C for 72 hours, and then checked for infectivity in the same manner into calf 151. Blood only was frozen at -20°C and -65°C and checked for infectivity in calves 155 and 153 respectively. Blood was stored unfrozen at 5°C for 72 hours then checked for infectivity in calf 156.

Results: Results are tabulated in Table II. Freezing at -20°C without DMSO destroyed infectivity. The addition of DMSO was obviously useful in maintaining infectivity. A -65°C was preferable to -20°C, although for 72 hours 5°C gave the shortest incubation time.

TABLE II

Storage of *A. marginale* Infected Blood with and without DMSO.

Treatment	Temperature °C	Recipient Animal	Incubation Time	Maximum Parasitemia
Blood + DMSO	-20	154	38 days	<0.1%
Blood + DMSO	-65	151	20 days	14%
Blood only	-20	155	No Anaplasma response	
Blood only	-65	153	38 days	1.0%
Blood only	5	156	17 days	9.0%

Vaccine Experiment #3

It would be desirable to have a standard inoculum producing infection with a consistant incubation time. If this could be developed, premunition with virulent Anaplasma would be feasible since the optimum time of treatment could easily be judged.

Materials and Methods:

A total of 41 ml citrated blood from calf 174 having a PCV of 19% and a parasitemia of 44% was mixed with 82 ml blood from calf 176 having a PCV of 23% and a parasitemia of 40%. The total volume of 123 ml was mixed with an equal amount of 6% DMSO in 0.85% NaCl and dispensed in 2 ml aliquots and frozen at -65°C by placing in the Revco deep freeze. The blood was checked for infectivity by inoculating 1 ml of the DMSO-Blood mixture subcutaneously in a splenectomized calf at 7, 36, and 64 days.

A second pool of blood was collected from deer 165 at a time when the PCV was 22% and the parasitemia 25%. In this instance the blood was mixed with equal parts of 4 molar DMSO (7.8%) in 0.85% NaCl, after which 1.4 ml aliquots were measured out and frozen at -65°C. This blood has been checked for infectivity by injecting 1 ml subcutaneously into a splenectomized calf before freezing, 16, 36 and 68 days after freezing.

Results: The results to date are presented in Table III. There appears to be a gradual increase in incubation time associated with the increase in storage time. These trials will continue in the hope that a fixed incubation time will evolve, which can be relied upon.

TABLE III

Effects of Storage at -65°C on Infectivity and Incubation Time.

Source	Length of Storage	Incubation Time	Low PCV	High Parasitemia
Bovine	7 days	31 days	7%	15%
Bovine	36 days	32 days	11%	17%
Bovine	64 days	39 days	9.0%	17%
Deer	0	15 days	7%	50%
Deer	16 days	31 days	9%	22%
Deer	36 days	40 days	10%	40%
Deer	68 days	- Incomplete -		

Vaccine Experiment #4

An effort was made to grow A. marginale of deer origin in Angora goats. The goat is inexpensive, and readily available in Texas, so was considered a possibility as an experimental animal and also a foreign host which if suitable might prove satisfactory for attenuating the A. marginale organism. The injection of a splenectomized goat with A. marginale infected deer cells failed to produce any evidence of parasitemia or infection. A sub-passage from this goat to another goat and to a splenectomized calf were equally unproductive. It was concluded that this animal was not suited to sustain Anaplasma.

Experiment #5

Recent literature cites the use of corticosteroids to enhance Babesia parasitemia. It was decided therefore to try this drug on Anaplasma carrier calves to determine if relapses and increases in parasitemia might occur following steroid therapy, and also to determine the influence of such therapy in propagating Anaplasma in splenectomized sheep. A preliminary trial to evaluate the influence of this treatment on total leucocyte counts in sheep was undertaken.

Materials and Methods

Azium (a product of Schering Corporation) brand of dexamethasone was used in these trials. Chemically it is 9-alpha-fluoro-16 alpha-methyl-prednisolone. Modification of the basic corticoid structure as achieved in Azium offers the advantage over older corticosteroids of greatly enhanced anti-inflamatory effects with lower dosages.

Three splenectomized Anaplasma carrier calves were given 3 injections at the rate of 0.05 mg/kg on a Wednesday, Friday and Monday. Three similar calves served as controls. Parasitemia and PCV values were recorded approximately 30 days before and after treatment.

Influence of Azium on WBC was recorded in sheep. Four sheep, 2 of which were splenectomized were injected I.M. Wednesday, Thursday, Friday and Monday with 0.05 mg Azium/kg. Four similar sheep, 2 of which were splenectomized served as controls. Total leucocyte counts were made on an electronic counter (Fisher's Autocytometer) daily during the injection period.

Results: Group averages of treated and non-treated calves are presented in figures 8 and 9. Azium produced no detectable changes in either PCV or parasitemia of carrier calves, at the average dose levels employed. Results of WBC counts are tabulated in Table IV for both treated and non-treated sheep.

No significant differences between total WBC of the treated and non-treated groups could be detected.

FIGURE 8

Influence of Azium on PCV of Infected Calves.

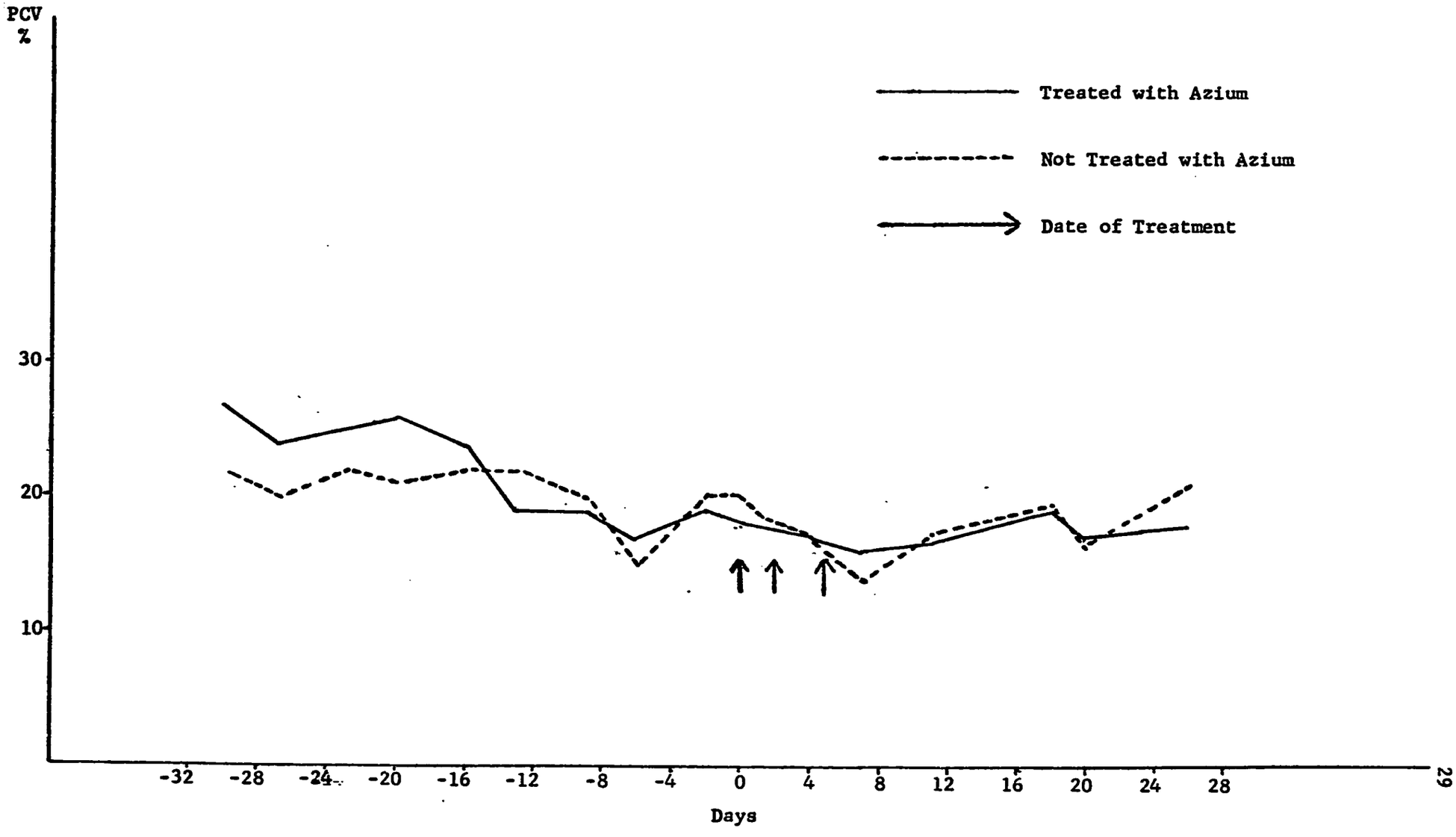


FIGURE 9

Influence of Azium on Parasitemia of Infected Calves.

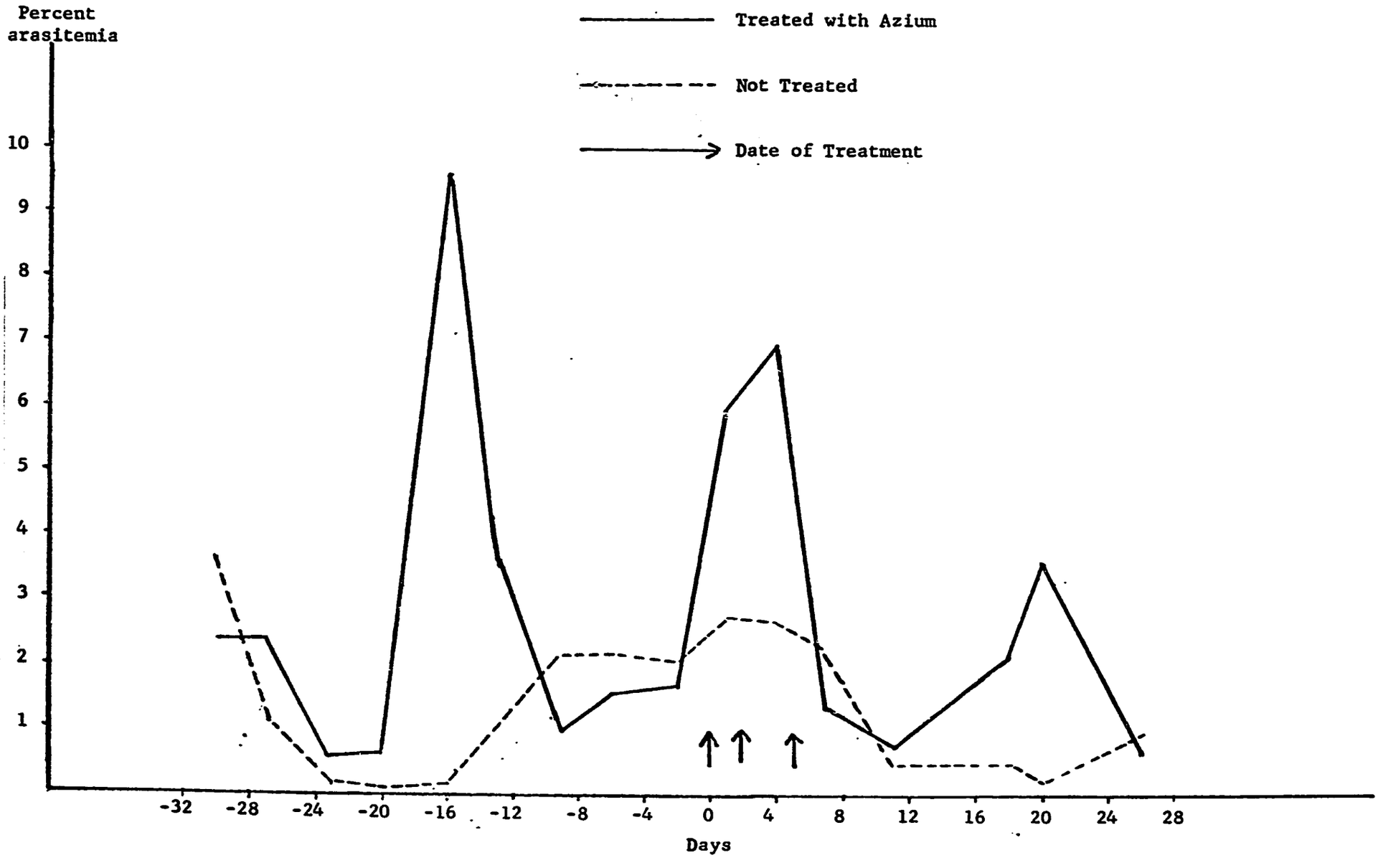


TABLE IV

Average WBC

	Number of Animals	Pre Azium	Day of 1st Injection	24 hrs.	48 hrs.	5 days	8 days
Not Treated with Azium	4	7.67	6.5	6.62	5.75	4.8	7.48
Treated with Azium	4	7.57	7.1	7.82	7.58	4.09	7.32
Significance Value of F		NS <1.0	NS <1.0	NS 1.96	NS 5.37	NS 2.35	NS <1

A marked drop occurred in the treated group 5 days after onset of treatment which if compared only to previous counts on the same animals would probably have been significant. A similar unexplained drop occurred among the controls which effectively nullifies this observation.

Vaccine Experiment #6

Serial sheep passage has just been started. Two splenectomized sheep have received identical inoculums. One sheep has been receiving Azium at doses about double those described in experiment 5. Observations are in progress, and serial passage will be pursued using the technique capable of producing the highest parasitemia.

NUEVO LAREDO - EXPERIMENT #1

Boophilus annulatus As A Vector for Anaplasmosis in Cattle and White-tailed Deer.

A partially engorged Boophilus annulatus female designated SpD_2 was recovered December 12, 1969 from a deer in Dimmit County, Texas. A 75% larval hatch was recorded January 1, 1969 from this female. On January 31, 1969 these larvae (SpD_2F_1) were placed on an intact calf 414 of local origin (Nuevo Laredo), with an unknown history of possible hemo-parasite infection. Engorged female ticks were recovered March 1, 1969. A sizeable number of larvae designated SpD_2F_2 were recovered. These larvae (SpD_2F_2) were used in trials to determine their ability to transmit hemotropic infections to 2 splenectomized calves and 2 intact deer.

Materials and Methods

Each of 2 splenectomized calves, free of anaplasmosis and babesiosis, identified as 480 and 185 were infested on day 0 with 1 gram SpD_2F_2 larvae.

Each of 2 white tailed deer (Odocoileus virginianus) identified as 471 and 475 were infested on day 0 with 1 gram of SpD_2F_2 larvae.

On day 7, 14, 21, 42, and 70, 10 ml of blood was taken from each of the 2 deer and inoculated subcutaneously into splenectomized calves free of anaplasmosis and babesiosis. Calves 172 and 178 were

inoculated with blood from deer 475 and 471 respectively.

Observations of all animals were made at weekly intervals, and included packed cell volumes (PCV) Giemsa stained blood smears, and complement-fixation (CF) tests for anaplasmosis.

Results

Calves 480 and 185 died of acute anaplasmosis, 65 and 68 days respectively after larvae were released on them. Calf 185 developed a 40% A. marginale parasitemia and a positive anaplasmosis CF test prior to death. Calf 480 developed a 64% A. marginale parasitemia and a positive anaplasmosis CF test prior to death. Packed cell volumes were markedly depressed, but low PCVs were not determined due to the interval between observations.

Deer 471 and 475, exposed to the same generation larvae of common origin failed to show evidence of anaplasmosis, in either blood smears or the CF test. Anaplasma marginale could not be recovered from either deer by blood inoculations in splenectomized calves. Claves 172 and 178 were observed seventy days after the last blood inoculation, and neither showed evidence of anaplasmosis or other hemoparasitic infection. Deer 471 was held in a wire enclosure during the tick feeding period and a large number of engorged females were recovered. Deer 475 could not be adequately restrained during the feeding stage, and as a result managed to pick off all ticks. No engorged females were recovered from 475, although nymphal stages were observed.

Conclusions:

The failure of white tailed deer to develop evidence of anaplasmosis following exposure to known *Anaplasma* infected *Boophilus annulatus* ticks suggests that deer are not an important reservoir of infection where *Boophilus annulatus* is the principle vector. It is presumed that the ticks recovered from Texas were infected during the feeding on calf 414 at Nuevo Laredo. Transovarial transmission with *B. annulatus* of *A. marginale* to susceptible calves did occur.

Boophilus infested deer crossing the Mexican border are capable of introducing ticks which in turn were shown capable of transovarial transmission of anaplasmosis. In effect when these ticks are introduced an additional vector for anaplasmosis becomes prevalent, and capable of compounding disease problems.

Acknowledgement is made to Dr. O. H. Graham, Entomologist and Investigations Leader, Kerrville, Texas, and Mr. J. L. Trevino for their generous cooperation and assistance.

DIMMIT COUNTY

BOOPHILUS ANNULATUS COLLECTED FROM A DEER

ENGORGED FEMALE SpD_2

↓

LARVAL HATCH

1 GRAM LARVAE

↓

CALF 414 (NUEVO LAREDO)

↓

ENGORGED FEMALE

↓

1 GRAM LARVAE

SPLENECTOMIZED CALF 480	SPLENECTOMIZED CALF 185	DEER 475	DEER 471
↓	↓	↓	↓
DIED OF ACUTE ANAPLASMOSIS	DIED OF ACUTE ANAPLASMOSIS	NEGATIVE FOR ANAPLASMOSIS	NEGATIVE FOR ANAPLASMOSIS

Environmental Influences on the Course of Anaplasmosis in Splenectomized Calves

Previous studies in Colombia revealed significant differences in the severity of anaplasmosis attacks occurring among animals of comparable age and breed, but located at different stations of varying climatic conditions. Generally infections were more severe in tropical low lands than those occuring in the Bogota area. This difference was tenatively attributed to the mean temperature or to the altitude, or possibly to a combination of factors. An attempt was made at Texas to vary temperature in an attempt to see if the severity of infection would alter accordingly.

Materials and Methods

Six splenectomized calves 4-6 months of age were divided into 2 groups July 9. One group was placed in an air conditioned room where the temperature range did not exceed 10 degrees and the average high for the entire period of test was $71.7^{o}F \pm 1.4$ and the average low was $66.4^{o}F \pm 2.9$. The second group remained in our experimental animal holding barn which is of metal construction and except for 10ft. chain link fencing around the perimeter and the individual pens is entirely open. The average high for the 75 days of testing (July 9 to September 22) was $96.1^{o}F \pm 6.2$ and the average low was $77.0^{o}F \pm 3.8$. The mean temperatures for those indoors was $69.0^{o}F \pm 3.5$, and those outside $86.5^{o}F \pm 10.9$.

All six calves were inoculated identically with Anaplasma infected blood on July 21. The inoculum consisted of 5 ml of blood from a splenectomized carrier calf with a parasitemia of 0.1%.

From the time of the first evident parasitemia until the animals were starting to recover 21 days later the average maximum outside temperature was 99.3°F ± 5.9, and the minimum 79.4°F ± 2.1 for a mean of 89.3. The average maximum inside temperature was 72.2°F ± 1.1, the minimum 67.6°F ± 1.7 for a mean of 69.9.

Observations for evidence of anaplasmosis and anemia were made on the six animals at least twice a week during the experiment.

Results

A summary of the comparative reactions is given in Table V. No significant differences are apparent. The group average PCV, parasitemia and, mean temperatures are shown in Figures 10, 11, and 12. No striking differences in the severity and course of the infection are detected.

Conclusions

The apparent lack of difference in response to anaplasmosis under different temperature conditions suggests that other factors may have been involved in observations made in Colombia. It is planned to repeat this experiment, in order to have numbers which will be statistically more valid. The major differences in anaplasmosis severity in Colombia was noted between Bogota and Cali where the mean temperature is 57.2°F and 75°F respectively.

This was considerably different than the conditions we were able to recreate in Texas, eventhough the temperature difference was almost the same. No evidence is presented in this study to support the hypothesis that the severity of anaplasmosis is associated with ambient temperature.

TABLE V

Environmental Influence on the Severity of Anaplasmosis in Splenectomized Calves.

	Average Mean Temp.	Pre Infection PCV	Incubation Time	High Parasitemia	Low PCV	High CF
3 calves kept indoors	69°F	30.3	18.3 days	39.3	9.3	1/254
3 calves kept outdoors	86.5°F	30.6	18.3 days	40.3	9.6	1/403

No significant differences were demonstrated.

FIGURE 10

Parasitemia and PCV values of Animals Maintained Inside at a Mean Temperature of 69°F.

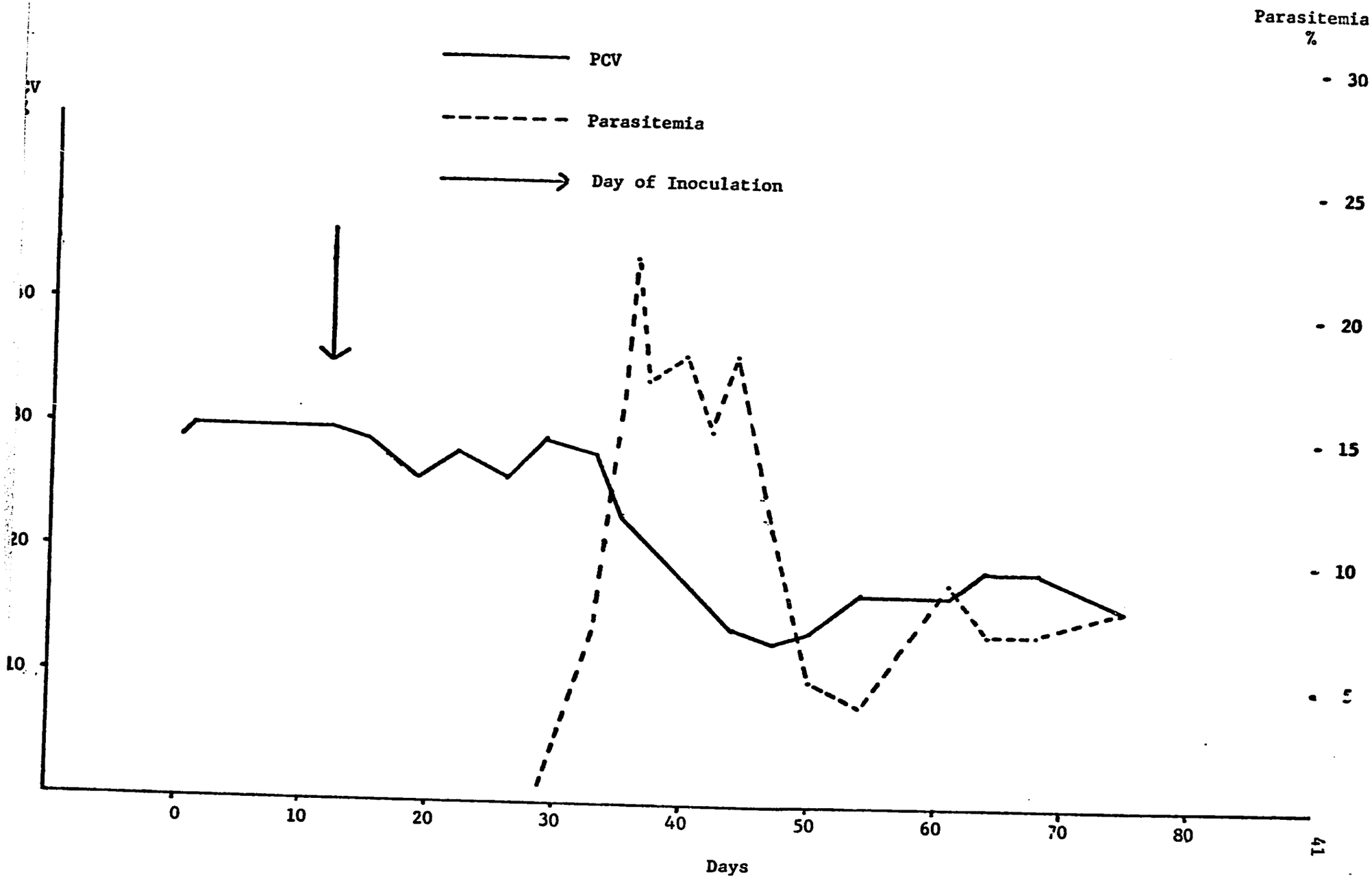


FIGURE 11

Parasitemia and PCV Values of Animals Maintained Outside at a Mean Temperature of 86.5°F.

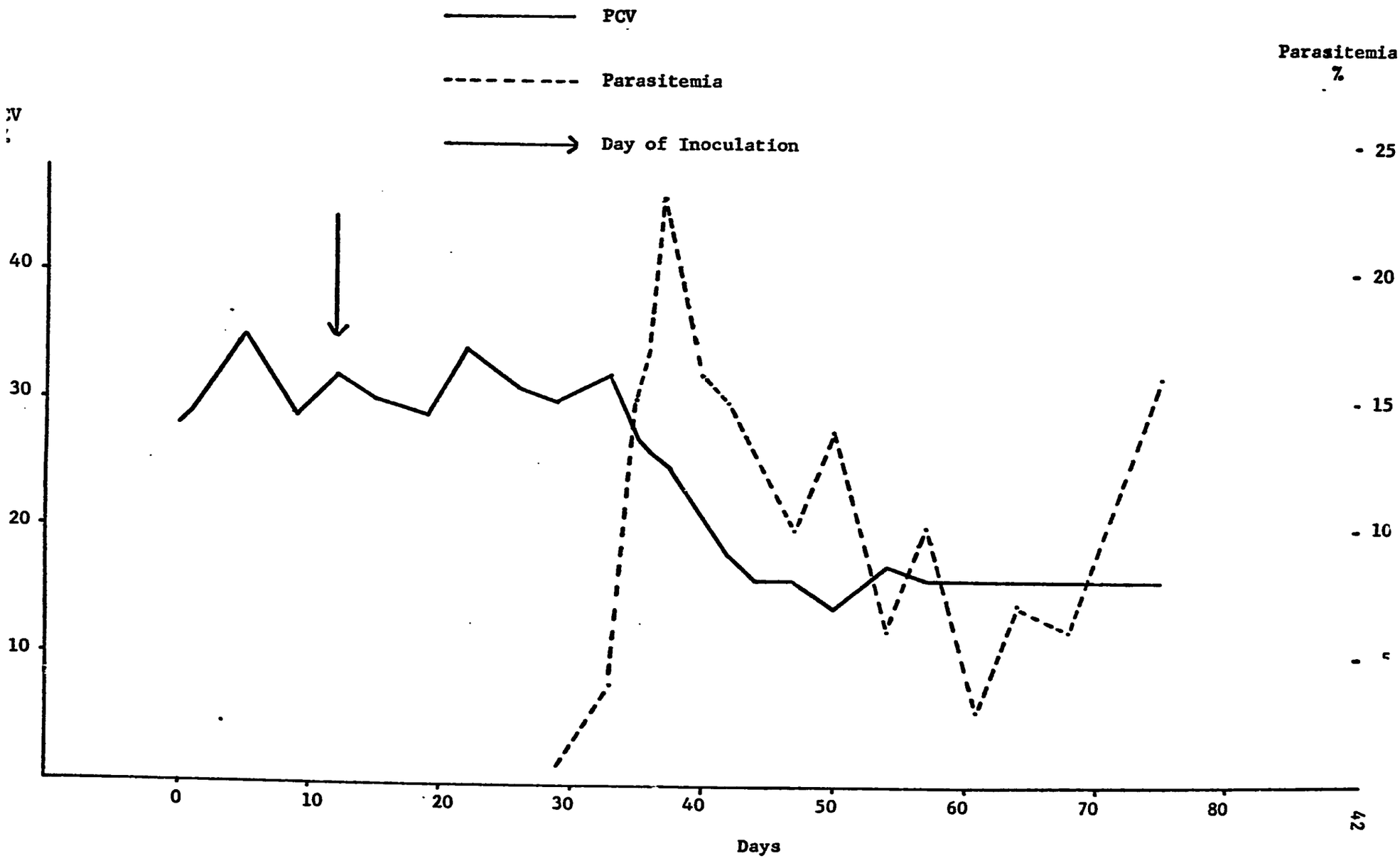
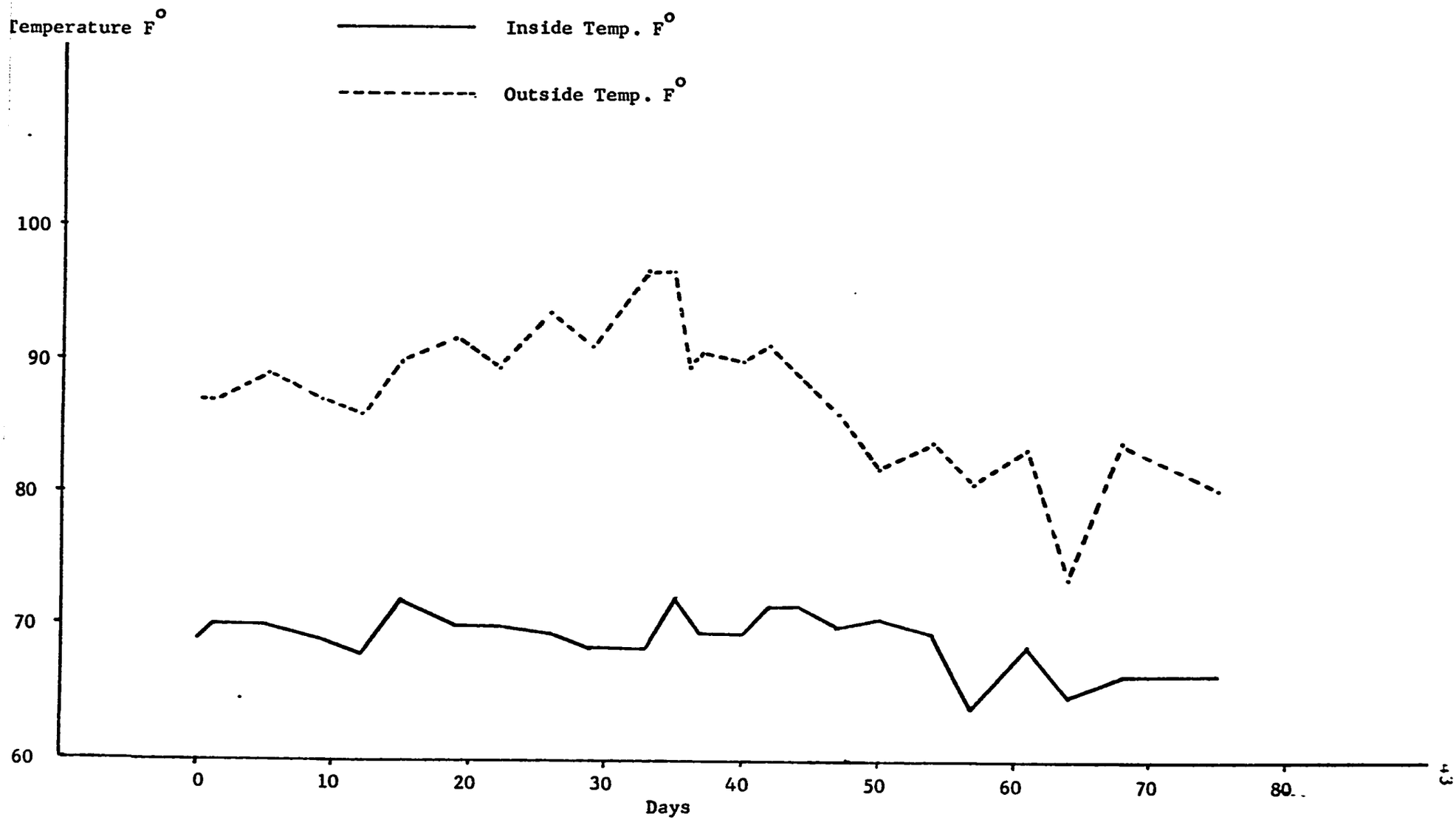


FIGURE 12

Mean Temperatures



Babesia

Eventhough the cattle Babesias do not occur in the U.S., interest remains high due to the prevalence of this infection in Mexico. The border counties of Texas are under constant surveilance for the accidental introduction of Boophilus (the principle vector of babesiosis) from Mexico. Boophilus ticks are occasionally picked up in the U.S. eventhough cattle being imported from Mexico are dipped before crossing the border.

A few years ago a Babesia specie was identified in Texas white tail deer. This is a highly pathogenic organism and is responsible for deer die offs in some area. When transferred to splenectomized deer it has characteristically produced death in 4 to 7 days in about 90% of the deer. Attempts to transmit this pathogen to cattle, sheep and goats have been unsuccessful.

Our work on Babesia of ruminants has been limited by the absence of the cattle Babesias in the U.S., and by the shortage of experimental animals for the deer Babesia. Through a cooperative project with the U.S.D.A., some Babesia studies have been conducted using the vector Boophilus annulatus. A few limited therapeutic trials have been conducted in deer infected with Babesia cervi.

Babesia cervi Experiment #1

An effort was made to transfer Theileria from a presumed carrier axis deer into a splenectomized white tailed deer (164). The injection of infected blood was made January 20, 1969. On January 23 Babesia cervi appeared in the blood smears. On the 25th after the diagnosis of Babesia became certain, deer 164 was injected with 1.5 ml ganaseg I.M. at the rate of 3 mg/kg bw. Two days later Babesia parasites had disappeared. Only the occasional blood parasite was seen thereafter until 46 days after exposure, when a rapidly rising Theileria parasitemia was observed. This parasitemia reached a 21% level then slowly receded. The Theileria has persisted in deer 164 for almost a year without evidence of a Babesia infection.

The object of this experiment was to determine if the Babesia infection had persisted, eventhough not recognized on blood smear, and in the presence of a persistent Theileria parasitemia.

Method: On May 29, 10 ml of blood from deer 164 was injected I.V. into splenectomized deer 160. At the time of transfer deer 164 was showing a 6% Theileria parasitemia. On the same date splenectomized sheep 110 was injected with blood from 164. Two weeks following blood from sheep 110 was injected into splenectomized deer 163.

Results: Deer 160 showed a Babesia parasitemia 4 days after exposure, and died the day following from acute babesiosis. The signs of anemia and hemoglobinuria were marked. Sheep 110 showed no signs of infection. The blind transfer into deer 163, 2 weeks later also produced no signs of infection.

Conclusion: Babesia cervi was present in deer 164 notwithstanding the Theileria parasitemia, and the absence of Babesia positive blood smears. The absence of any Theileria parasitemia in deer 160 confirms the shorter incubation time for Babesia in deer. Babesia cervi is not able to grow or even to maintain itself in sheep.

Babesia cervi Experiment #2-Influence of Treatment and Pre-exposure to other Hemoparasites on Babesia Infection

Deer 164 shown in experiment #1 to be a Babesia carrier has been used in some of these studies as a source of infecting inoculums. Transfers have been made as late as November 21, showing 164 infective for as long as 6 months after the first transfer reported in experiment #1.

Object: To determine the influence of prior infection with Anaplasma, and Theileria on the course of Babesia infection. Secondly to determine the influence of treatment with the Burroughs Wellcome experimental drug 4A65.

Method: 1.) Splenectomized deer 125 was inoculated S.C. with 5 ml blood from deer 164. Deer 125 had no previous history of other blood parasites and was considered free of such infections. No treatment was administered.

2.) Splenectomized deer 170 was inoculated S.C. with 15 ml blood from a deer in East Texas suspected of having a Babesia infection. Deer 170 had no previous history of other blood parasites and was considered free of such infections. No treatment was administered.

3.) Splenectomized deer 175 was inoculated I.V. with 10 ml blood from deer 163 which had previously been treated with 4A65. Deer 175 had no previous history of blood parasites and received no treatment.

4.) Splenectomized deer 126 was inoculated S.C. with 5 ml blood from deer 164. Deer 126 had no previous history of other blood parasites. Treatment with 4A65 was administered 1 time at the rate of 2 mg/kg bw., 4 days after infection when the Babesia parasitemia was 4%.

5.) Splenectomized deer 163 was inoculated I.V. with 10 ml blood from deer 164. Deer 163, eventhough inoculated with sheep blood 4 months previously had shown no diagnostic evidence of blood parasites. Treatment with 4A65 was administered 1 time at the rate of 1 mg/kg bw, 2 days after infection when the Babesia parasitemia was 1.5%.

6.) Splenectomized deer 159 was inoculated I.V. with 10 ml blood from deer 164. Deer 159 has been infected with Theileria cervi, and Anaplasma marginale and had apparently recovered from the acute phases of both these infections. At the time of exposure to Babesia a 0.3% Theileria parasitemia was present, but the CF response to Anaplasma was negative. Treatment with 4A65 was administered 1 time at the rate of 4 mg/kg bw 2 days after infection when the Babesia parasitemia was 2.0%

7.) Splenectomized deer 161 was inoculated I.V. with 10 ml blood from deer 164. Deer 161 has been infected with Theileria cervi and Anaplasma marginale. Deer 161 had proven relatively refractory to Theileria with only a 3% parasitemia developing, and no pronounced drop in PCV. Following exposure to Anaplasma a marked parasitemia of 31% developed and this was followed by a low PCV of 13%. A 1/160 Anaplasma CF titer existed at the time of Babesia exposure. No treatment was administered after Babesia exposure.

8.) Splenectomized deer 165 was inoculated S.C. with 5 ml blood from deer 164. Deer 165 has been infected with Theileria cervi and Anaplasma marginale. Deer 165 had reacted severely to Theileria developing a 48% parasitemia which was associated with a 12% PCV. The reaction to anaplasmosis was also moderately severe with a parasitemia of 27% occuring which was associated with a 13% PCV. These 2 infections were 45 days apart with a period of apparent recovery in between. The CF response immediately following Babesia exposure was 1:10. No treatment was administered after Babesia exposure.

Results: The results are summarized and tabulated in Table X.

Figures 13, 14, 15, 16, 17, 18, and 19, illustrate the PCV and parasitemia response of each animal.

TABLE X

Response of Splenectomized Deer *Babesia cervi* Infections

		Incubation Time	High Recorded Parasitemia	Low PCV	Death	Time of Death
I	125	3 days	92%	4%	X	6 days
	170	4 days	15%	10%	X	8th day
	175	4 days	14%	14%	X	6th day
II	126	4 days	6.0%	18%	-	-
	163	2 days	10.0%	10%	-	-
	159*	2 days	NT	NT	X	NT
III	161	10 days	1.0%	18%	-	-
	165	3 days	15%	<20%	X	7th day

I - Control animals - Not treated and no history of previous infection.

II - Treated 1-2-4 mg/kg bw - 4A65.

III - Not treated, but had a previous history of both *Anaplasma* and *Theileria* infection.

*-159: Died following treatment with 4A65, when drug was administered at the rate of 4 mg/kg bw.

NT: No Test or Not Applicable.

Results: Treatment using 0.1 mg and 0.2 mg/kg bw of 4A65 while not eliminating infection did reduce the severity sufficient to allow the animal time to recover. Treatment with 0.4 mg/kg bw of 4A65 was in itself toxic. Deer 159 died within 10 minutes of the treatment. Severe respiratory depression was noted. Artificial respiration was unsuccessfully administered.

Conclusions: Deer with no previous history of hemoparasites, and in the absence of treatment died in 6-8 days after exposure, following typical signs of babesiosis.

The reasons for the survival of deer 161 are not clearly known. Infection obviously developed but did not proceed in the accustomed pattern. Deer 161 had previously failed to react severely to a Theileria infection, suggesting a possible inherent resistance factor. Deer 161 reacted markedly to anaplasmosis and developed a high CF titer prior to Babesia infection. This may also have been a factor. Until this work is repeated some difficulty can be expected in evaluating the possible influence of previous hemoparasite infection on infections produced by a different organism. Deer 165 eventhough having a history of previous infections died in the normal time.

Treatment with 4A65 appears highly effective when administered at 0.1 mg/kg or 0.2 mg/kg in the early stages of infection. Treatment stopped the very rapid progress of infection in both instances where it was tried. Extreme caution is important due to the toxic side effects produced by 4 mg/kg bw.

FIGURE 13

Splenectomized Deer 125 -- Induced *Babesia* Infection.
Day 0 - Date of Exposure

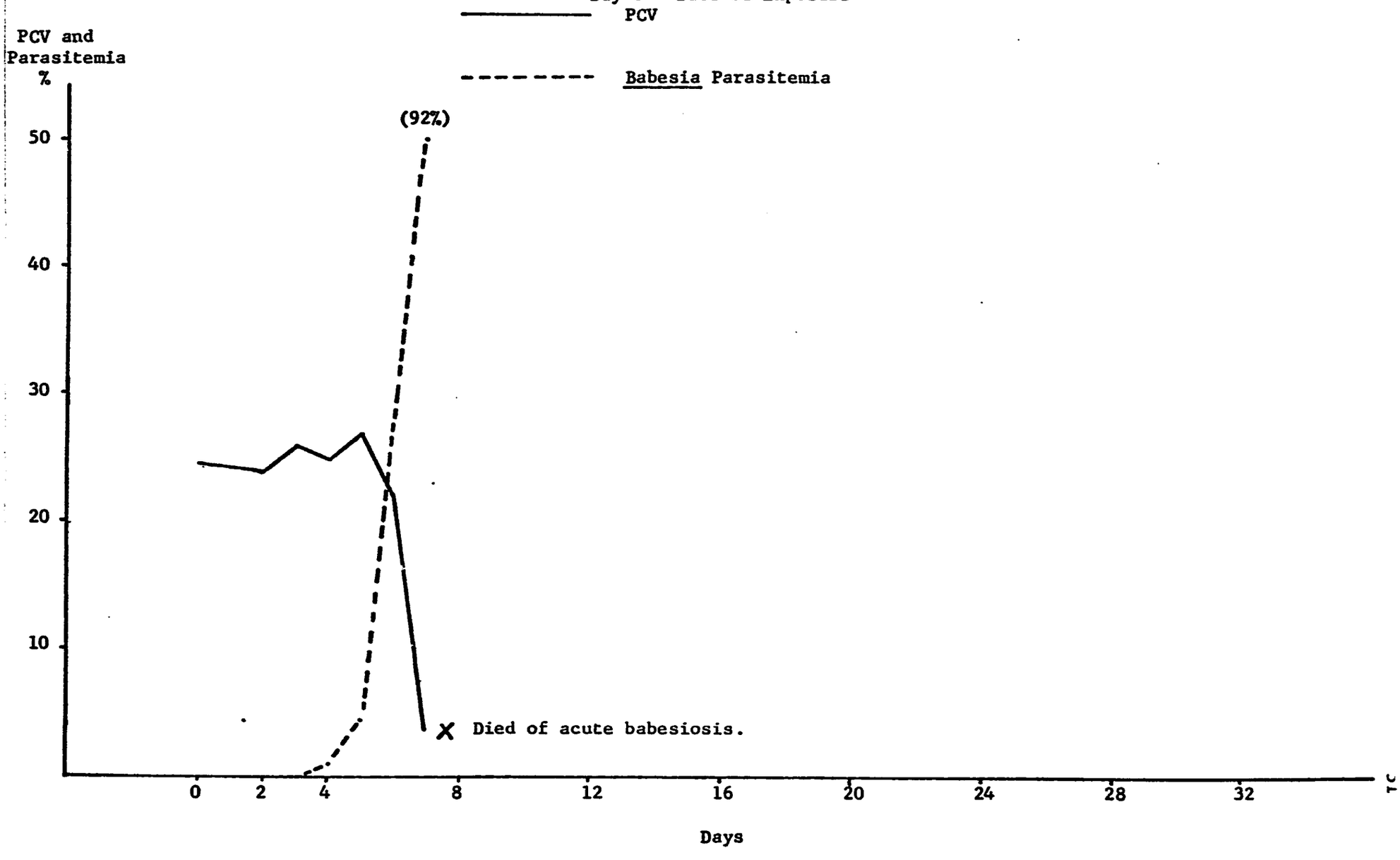


FIGURE 14

Splenectomized Deer 170 -- Induced *Babesia* Infection.

Day 0 - Date of Exposure

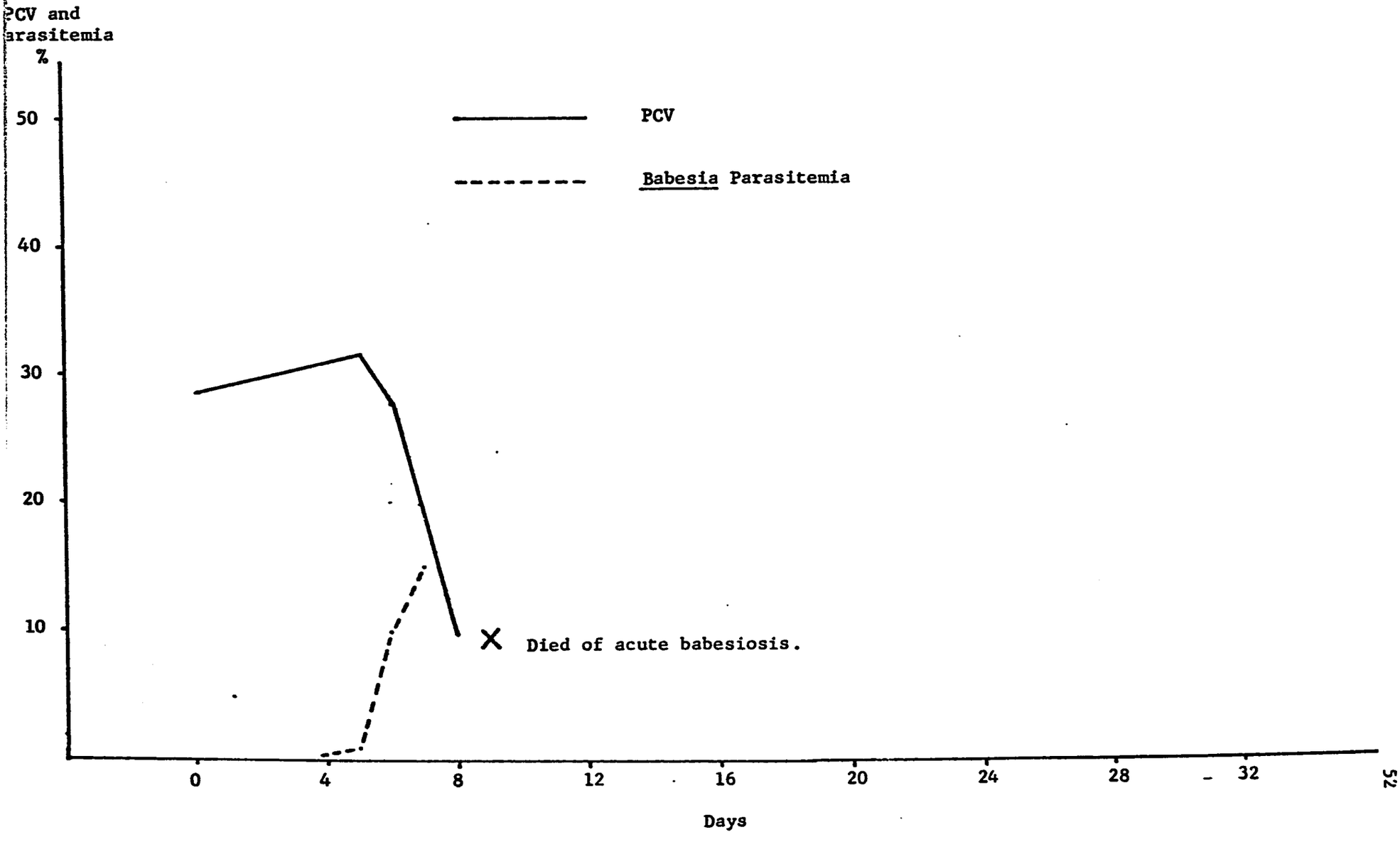


FIGURE 15

Splenectomized Deer 175 - Induced *Babesia* Infection.

Day 0 - Date of Exposure

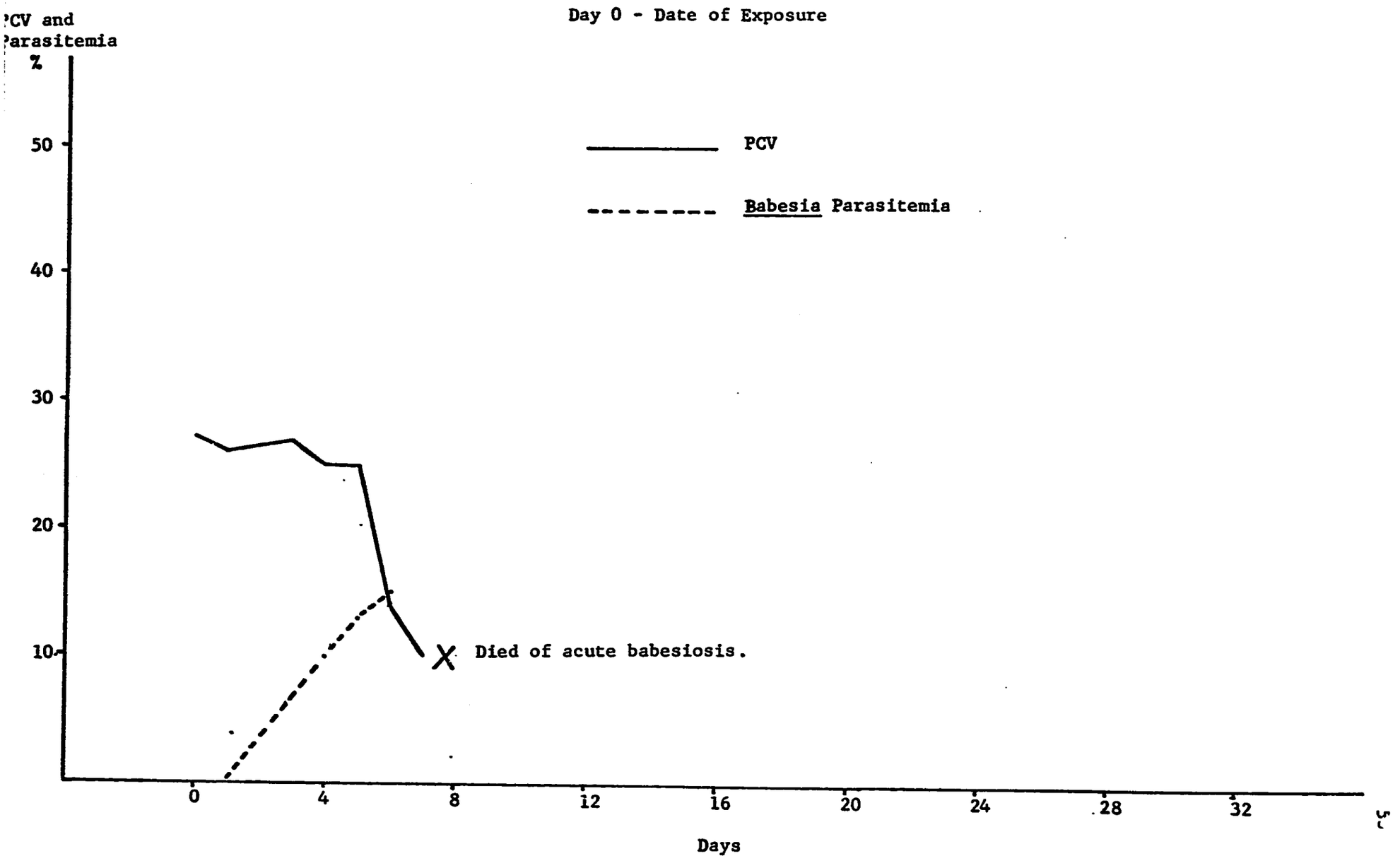


FIGURE 16

Splenectomized Deer 126 - Induced Babesia Infection Followed by Treatment (4A65).

Day 0 - Date of Exposure

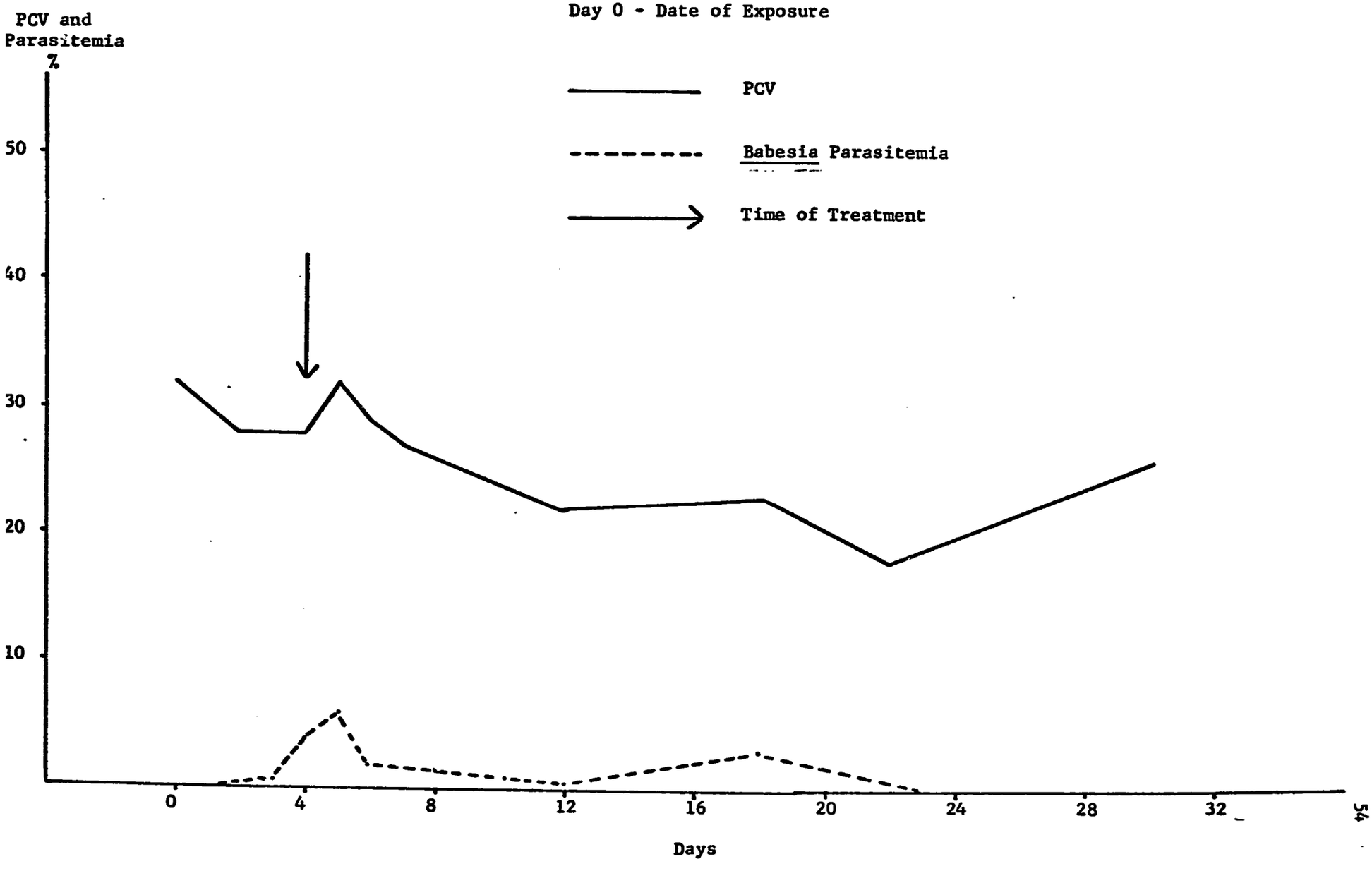


FIGURE 17

Splenectomized Deer 163 - Induced *Babesia* Infection Followed by Treatment. (4A65)

Day 0 - Date of Exposure

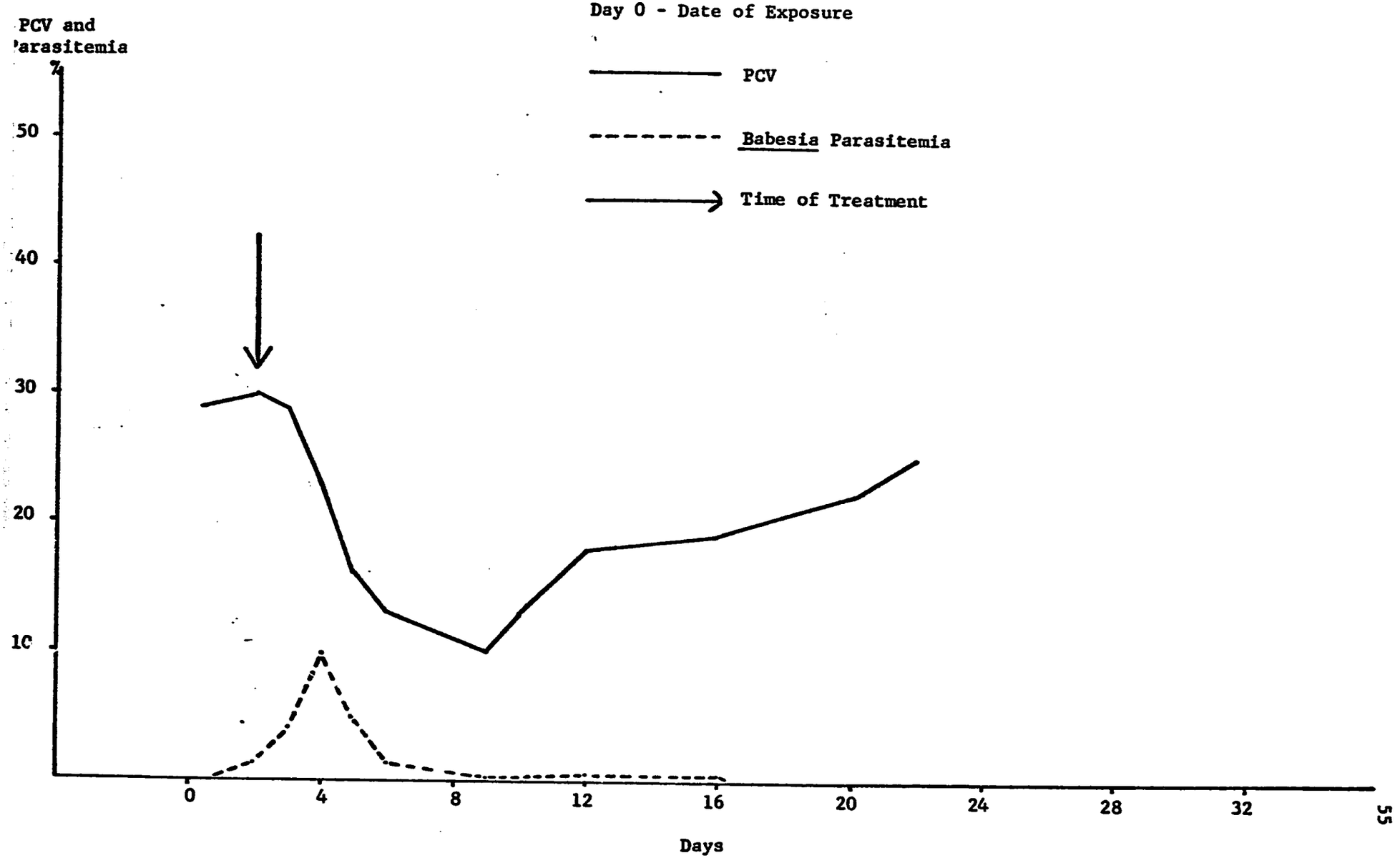


FIGURE 18

Splenectomized Deer 161 - Induced *Babesia* Infection in *A. marginale* and *T. cervi* Carrier Deer.

Day 0 - Day of *Babesia* Exposure

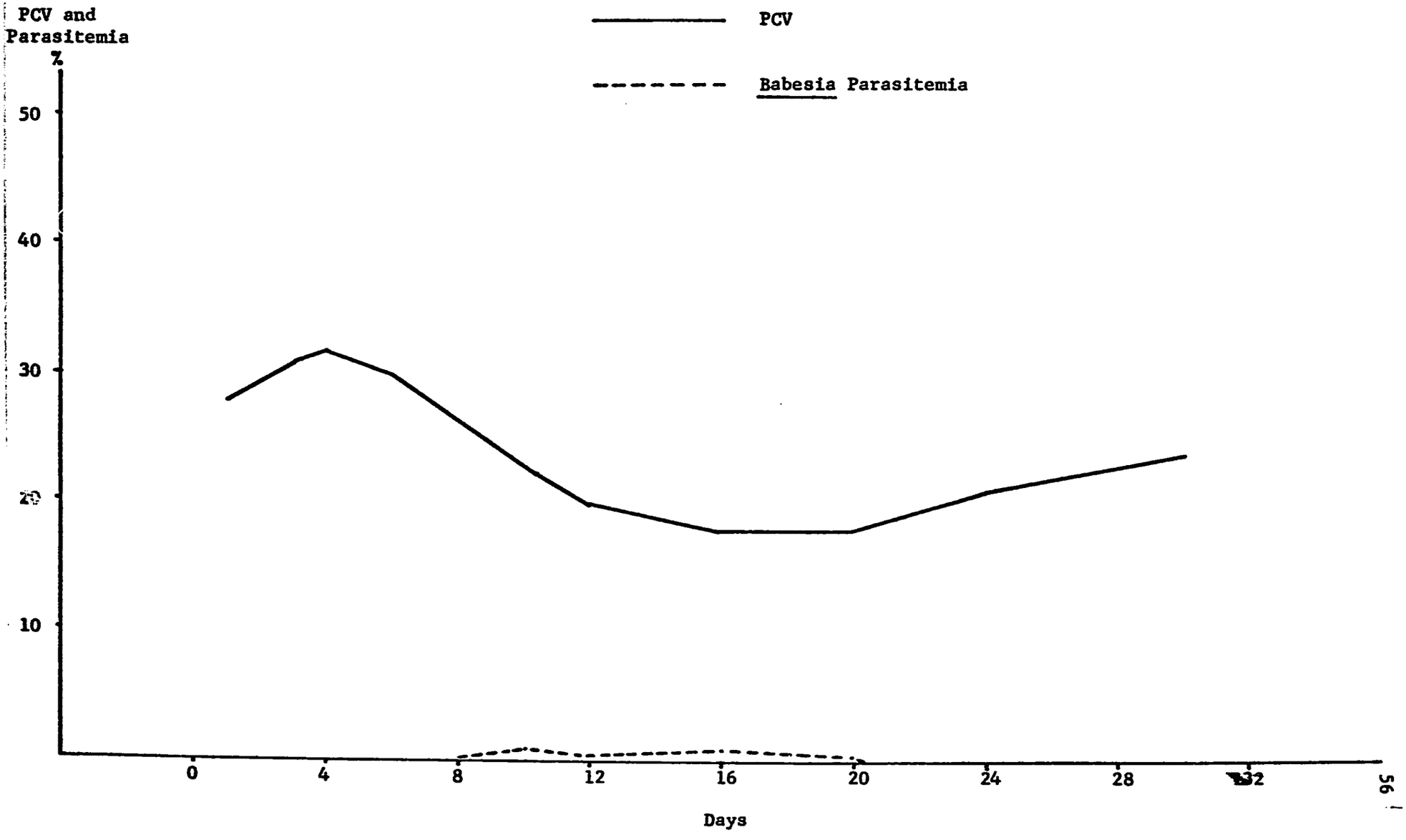
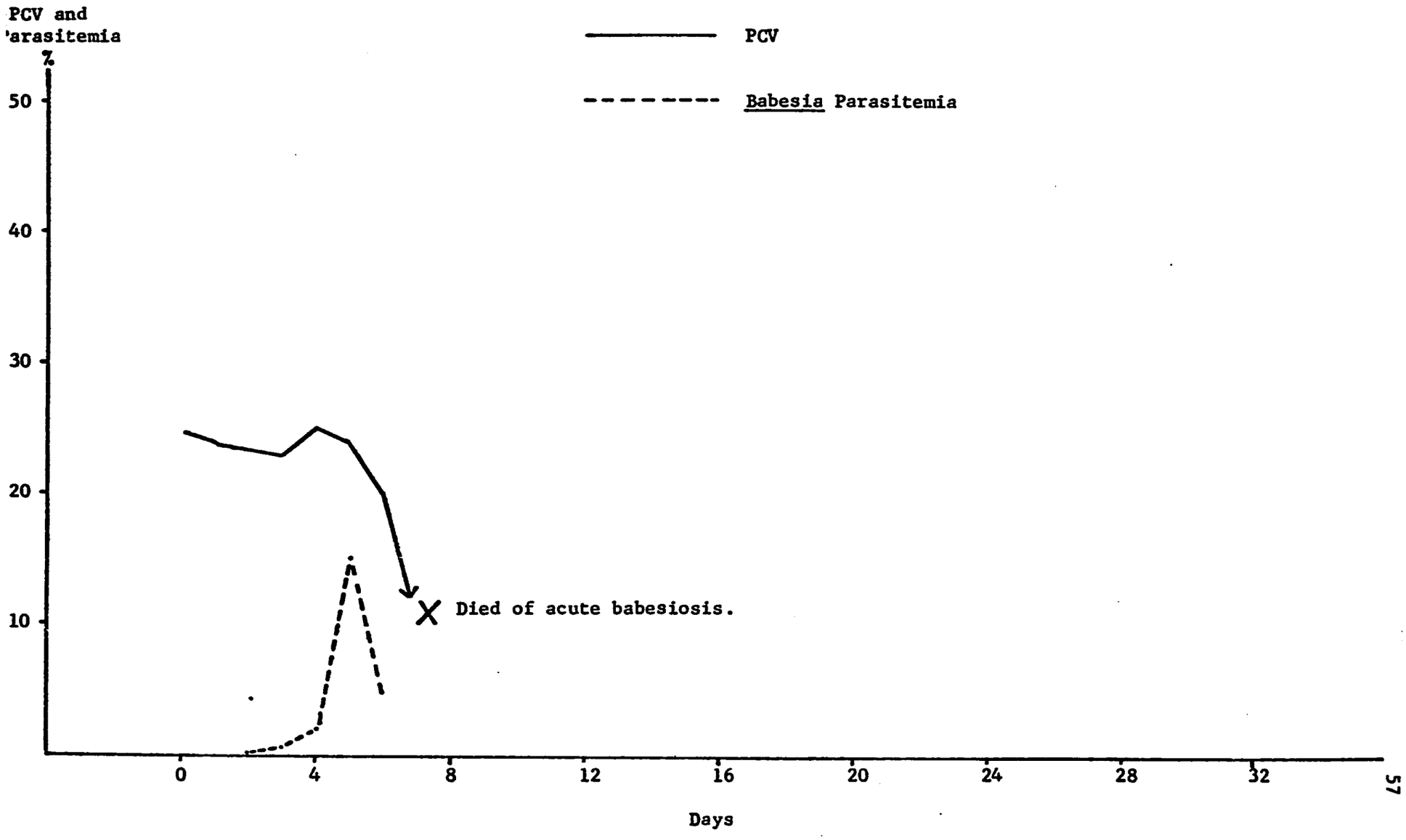


FIGURE 19

Splenectomized Deer 165 - Induced *Babesia* Infection in *A. marginale* and *T. cervi* Carrier Deer.

Day 0 - Day of *Babesia* Exposure



NUEVO LAREDO - EXPERIMENT #2

Efforts to Transmit Babesia to White Tailed Deer by Blood Inoculation and with Babesia Infected Boophilus annulatus Ticks.

The movement of Boophilus infested white tailed deer across the border into Texas has been noted ( ). The hazards of re-introducing Boophilus into the U.S. are obvious, but it is not known whether or not these ticks may also be carrying Babesias which occur in border areas of Mexico.

This study was made to evaluate the hazard of deer acting as a reservoir host for Babesia, or as a vehicle for the introduction of Babesia infected ticks.

Materials and Methods

Deer 1 was infested on day 0 with 1 gram Boophilus annulatus larvae thought to be carrying Babesia bigemina. On day 0 a splenectomized calf (187) was similarily infested with 1 gram of larvae from the same source as used for Deer 1.

On day 0 Deer 2 was injected subcutaneously with 10 ml citrated blood from a known Babesia carrier calf. On day 0 a splenectomized calf (177) was similarily infected by injecting, subcutaneously, 10 ml citrated blood from the same Babesia carrier calf.

An effort to demonstrate a possible Babesia parasitemia in deer was made by inoculating blood into splenectomized calves at various intervals after tick infestation and after injecting Babesia infected

blood. Ten ml blood from Deer 1 was injected subcutaneously into splenectomized calf 1 on days 7, 14, 21, 28 and 35. The same procedure was followed to check Deer 2 for a Babesia parasitemia in a splenectomized calf.

Deer 2 was splenectomized 8 weeks after exposure to Babesia by blood inoculation. A second injection of 10 ml blood from calf 477 was made 4 weeks following splenectomy. Following this second exposure of Deer 2 an effort to recover a possible Babesia parasitemia was made by injecting 10 ml of Deer 2 blood into a susceptible splenectomized calf (178) 7, 14, and 21 days after exposure.

Engorged B. annulatus female ticks were collected from Deer 1, 25 days after larvae were first released. One gram of larvae from these females were placed on splenectomized calf 182, 70 days after the females were collected to determine if the Babesia has persisted in ticks during 1 generation on the deer.

Observations on experimental animals were made every 7 days, and included packed cell volumes and giemsa stained blood smears. Weekly anaplasmosis complement-fixation tests were conducted on serum of all animals for the duration of these experiments.

Results

The Boophilus larvae placed on Deer 1 and calf 187 attached and in Deer 1 went through a normal life cycle, with engorged females being harvested. Deer 1 showed no evidence of Babesia infection. Calf 187 died of acute babesiosis on day 16. Babesia was first

observed in stained blood smears on day 14. Death was preceded by elevated temperature, hemoglobinuria, and severe anemia. Efforts to recover a Babesia from Deer 1 were unsuccessful.

The inoculation of Deer 2 with known Babesia infected blood failed to produce any evidence of infection. Calf 177 died of acute babesiosis 9 days after receiving a similar inoculum. Babesias were first observed in stained blood smears 7 days after infection. Death was preceded by elevated temperature, hemoglobinuria, and severe anemia. Efforts to recover a Babesia from Deer 2 were unsuccessful.

Attempts to demonstrate a Babesia parasitemia in Deer 2 following splenectomy and a second exposure were unsuccessful. No evidence of a Babesia parasitemia was seen on stained blood smears. Attempts at sub-passage to a splenectomized calf were negative.

No evidence of Babesia in calf 182 has been detected 41 days after the release of larvae from Deer 1.

Conclusion

These studies failed to demonstrate growth or survival of Babesia in deer. Ticks known to be infected with Babesia placed on a deer were not capable of transmitting Babesia after having completed a life cycle on deer. It is doubtful therefore if ticks crossing the border from Mexico on deer would be capable of transmitting babesiosis to susceptible cattle, unless they first undergo a life cycle on infected cattle. In the event that B. annulatus ticks feed on Babesia carrier cattle, imported from Mexico or elsewhere an infective vector

would then exist capable of transmitting babesiosis. Should such a situation occur in border areas of the U.S. the potential exists for an explosive outbreak of babesiosis in the highly susceptible European breeds found in the U.S.

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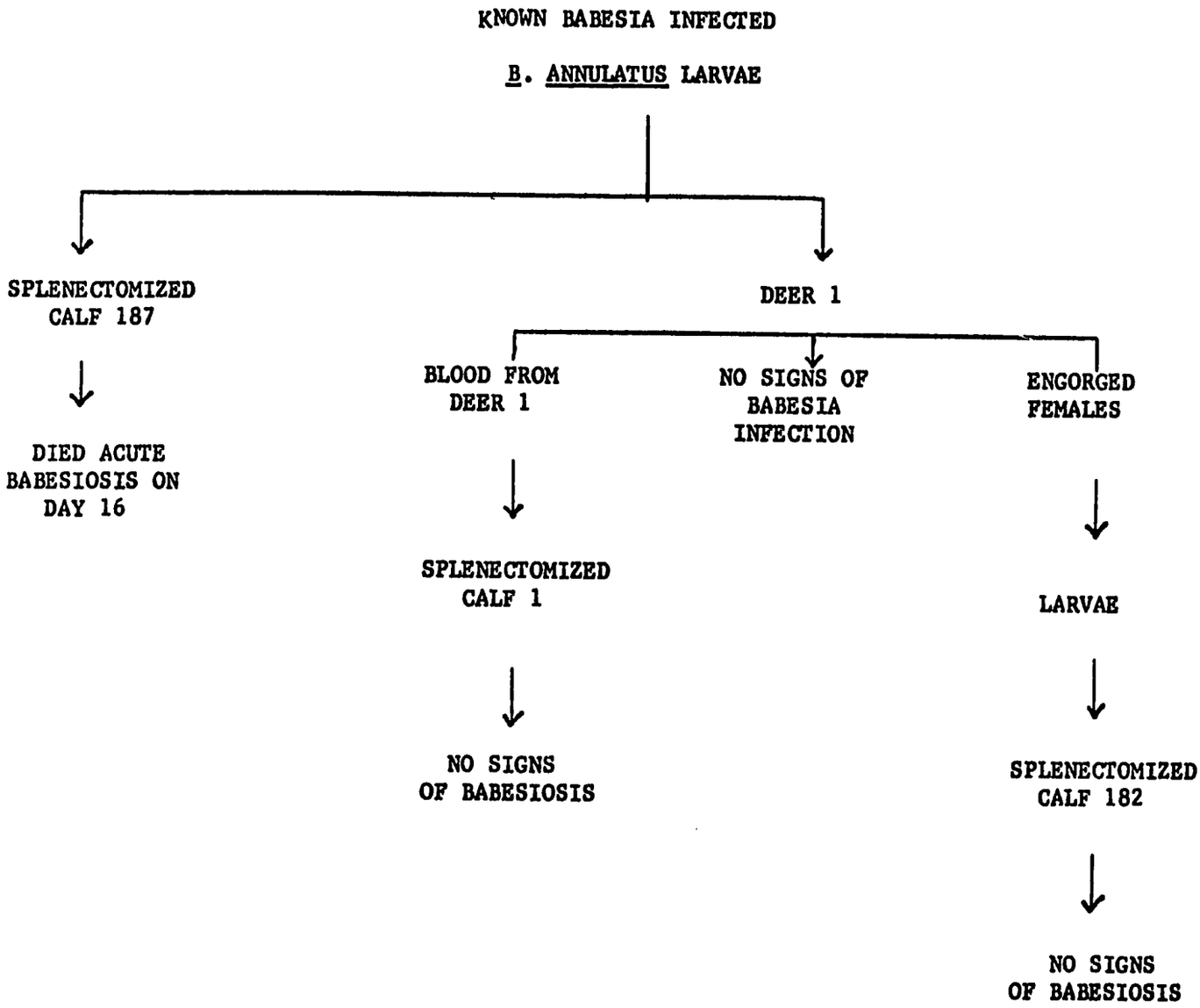

KNOWN BABESIA INFECTED
B. ANNULATUS LARVAE
SPLENECTOMIZED CALF 187
DIED ACUTE BABESIOSIS ON DAY 16
DEER 1
BLOOD FROM DEER 1
NO SIGNS OF BABESIA INFECTION
ENGORGED FEMALES
SPLENECTOMIZED CALF 1
NO SIGNS OF BABESIOSIS
LARVAE
SPLENECTOMIZED CALF 182
NO SIGNS OF BABESIOSIS

Babesia rodhaini

Babesia rodhaini, a highly pathogenic blood protozoa, in mice has proven a useful model by many workers interested in Babesia parasites of cattle. The many similarities between this organism and those occuring in cattle suggests that much basic work is possible with this specie not feasible with cattle. Many preliminary type studies have been undertaken to better understand the nature of this pathogen, what can be done with it etc. Initially we needed to know how best to pass the organism, what titers occur in blood, spleen and other tissues, what is the standard incubation time, what effects infection has on PCV, spleen size, and what levels of parasitemia occur etc., before immunization, and treatment trials can be fully interpreted.

Much of the work reported at this time will include such information, but the first of our studies on immunity have been made, and as time permits many more will follow.

A technique for heart puncture has been developed for securing blood samples for PCV, smear, and sub-passage, with a reasonable degree of success. Unless the mouse is to be sacrificed it is anesthetized using approximately 0.4 ml of a 1/10 dilution diabutol. The cardiac puncture is then made using a 1 ml tuberculin syringe equipped with a 5/8" 25 gauge needle. Heparin is drawn into the syringe and expelled. The residual heparin is sufficient to act as an anticoagulant. Generally 0.2 ml can be safely removed and this used to make a 1:10 dilution. Isotonic phosphate buffer solutions 0.01 M, pH 7.2 has been used routinely as a diluent. Titrations are generally made using 10 fold dilutions, LD50 being calculated as described by Reed and Muench.

For routine passage a 0.5 ml inoculum of 10^{-3} dilution of infected blood is made intraperitoneally (I.P.). This dilution is altered when challenging supposedly immune mice as will be indicated.

We have established a breeding colony of approximately 400 females, with a total population of about 1700 mice. Routinely on blood titrations only deaths are considered, since it becomes impractical to examine blood smears for the hundreds of mice sometimes on test. The nearly 100% mortality of untreated controls makes this approach feasible in evaluating titrations.

B. rodhaini Experiment #1

One of the first studies conducted was a titration to obtain some estimation of what titers occurred in mice, and the degree of parasitemias that could be expected.

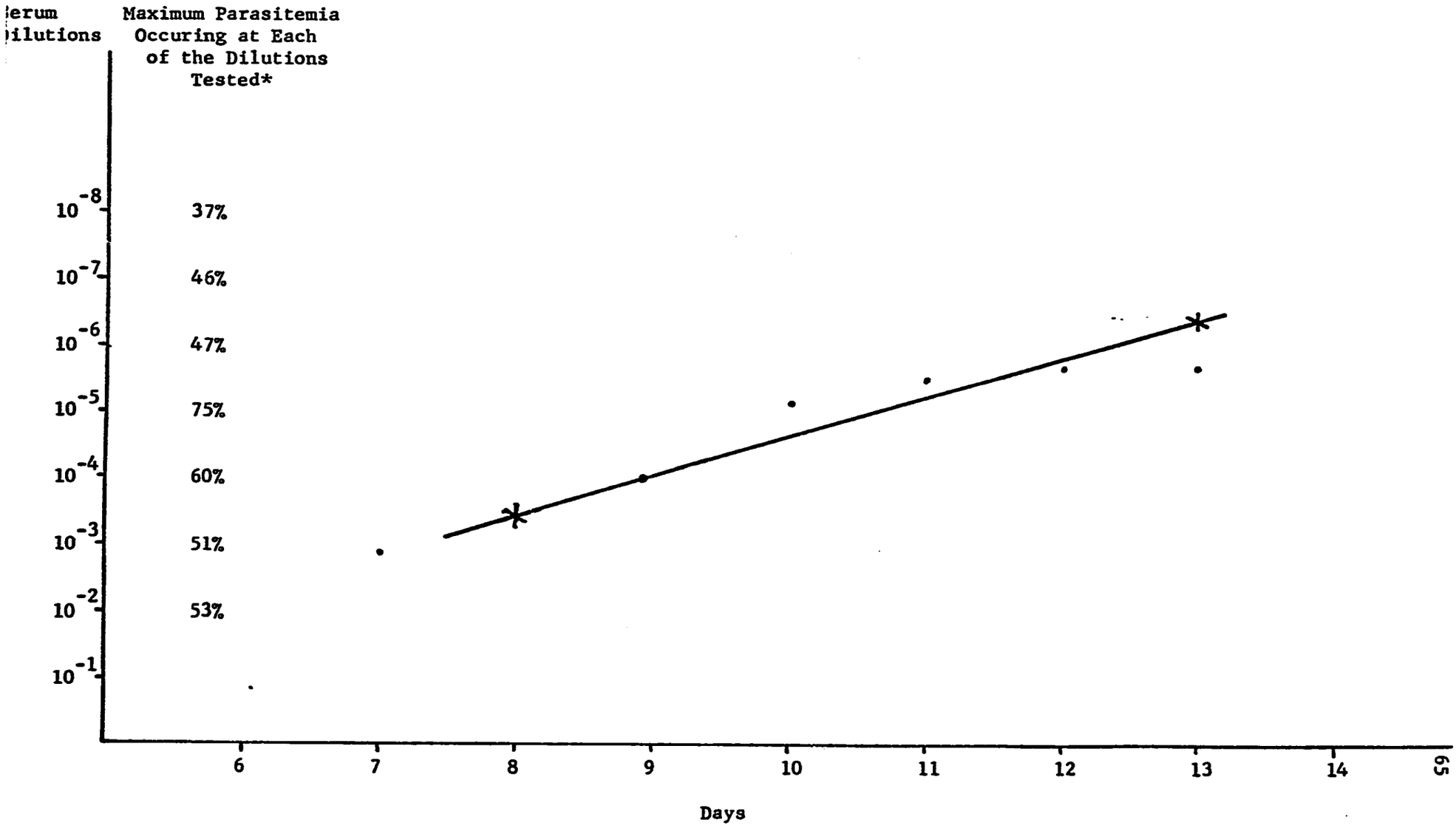
Method: Blood showing a 56%. Babesia parasitemia and a 24% PCV was diluted serially in 10 fold steps and checked for infectivity at 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}, dilutions. Five mice (approximately 35 grams) were used for each point. Parasitemias were made when mice were beginning to show evidence of infection, in each dilution. In addition to determining the LD50, the incubation time, or the time required for death to occur was plotted for each dilution.

Results: A LD50 titer of $10^{-7.625}$ was calculated for the titration. The time for death to occur increased progressively with the higher dilutions. A correlation was noted and a significant straight line regression was plotted in figure 20 . The parasitemias recorded in mice dying at 10^{-6-7-8} were not significantly less than mice dying at dilution 10^{-2-3} etc.

FIGURE 20

LD50 from Day 6 to Time of Maximum Titer at Day 14 for Babesia rodhaini.

*Differences are not significant.



Conclusion: Titers were higher than expected. Future titrations have included 10^{-9}. Small inoculums in anaplasmosis frequently result in longer incubation times. Some disagreement exist as to whether or not the severity of Anaplasma infection is altered by these small inoculums. All animals dying of Babesia rodhaini exhibited a parasitemia which was essentially the same at the high dilutions as seen in the lower dilutions. The evidence of this trial suggests that a longer incubation time does not influence the severity of the subsequent infection. In future studies incubation time must be considered in this light, and conclusion of altered virulence etc. cannot be made just because an increase in incubation occurs.

B. rodhaini Experiment #2

Object: To determine the influence of external factors on the survival of *B. rodhaini*. In anaplasmosis the infective particles cannot be thrown down by centrifugation at speeds up to 35,000 XG. Grinding will actually increase antigenicity of *Anaplasma* antigens. This trial was intended to determine the influence of freezing, grinding and centrifugation on *B. rodhaini*.

Method: A pool of 1:200 dilution of infected blood was used as source of all material. The first aliquot was frozen at -65°C, held 1½ hours then inoculated (0.5 ml I.P.) into 2 susceptible mice. A second aliquot was ground 1 minute at full speed in a Vir-Tis tissue homogenizer, and checked for infectivity in 2 mice. Two aliquots were taken from the ground material, one was centrifuged 10 minutes at 2,000 RPM at 5°C and then the supernate was checked in 2 mice for infectivity; the second was centrifuged 30 minutes at 35,000 XG at 5°C and then the supernate was checked in 2 mice for infectivity. Controls consisted of 2 mice injected with the 1:200 before any treatment.

Results:

				Deaths	Time Required
Control	1:200	-	No treatment	2/2	8 days
Frozen	1:200	-	-65°C - 1½ hrs	2/2	14 days
Virtis	1:200	-	Ground 1 minute	2/2	15 days
Centrifuge	1:200	-	2,000 RPM-10 min.	No Response	
Centrifuge	1:200	-	35,000 XG-30 min.	No Response	

Conclusion: Both freezing and grinding are detrimental to the infectious organism. Centrifugation even at low speeds was sufficient to remove infectious particles.

B. rodhaini Experiment #3

Object: To determine the effects of DMSO (dimethyl sulfoxide) with and without grinding as a preliminary to freezing experiments. Work with DMSO has indicated its value as an additive in Anaplasma infected blood being frozen and stored. Since a similar technique will probably be employed with B. rodhaini it is necessary to know if DMSO has any detrimental effects on the organism.

Method: A pool of infected blood from 2 mice showing 30% and a 31% parasitemia was used to prepare a 1:1000 (10^{-3}) dilution in 0.01 M PBS pH 7.2. A second 1:1000 dilution was prepared in 0.01 M PBS pH 7.2 to which 6% DMSO had been added. Half of each preparation was ground in the Vir-Tis 1 minute at full speed.

Titrations were conducted on each of the 4 preparations by making serial 10 fold dilutions in the respective diluents. Those preparations not ground were tested at $10^{-5,-6,-7,-8 \text{ and } -9}$, those ground in the Vir-Tis were tested at $10^{-3,4,5,6 \text{ and } 7}$. A total of 5 mice were used per point, and were injected with 0.5 ml I.P.

Results: End points were obtained for 3 groups. Only 1 of 5 mice died in the 4th group containing DMSO and ground in the Vir-Tis. The fact that 1 mouse died at 10^{-3} did suggest that the end point was not far from this dilution.

I - PBS - 0.01 M - pH 7.2, Final titer: $10^{-8.4}$

II - PBS - 0.01 M - pH 7.2 plus 6% DMSO, Final titer: $10^{-7.8}$

III - PBS - 0.01 M - ground in Virtis, Final titer: $10^{-3.3}$

IV - PBS - 0.01 M plus DMSO ground in Virtis, Final titer: $<10^{-3}$

Conclusions: The DMSO does have some detrimental effects on B. rodhaini, but probably not so much as to prevent its use as a preservative in future experiments. The effects of grinding in the Vir-Tis confirms experiment #2, and quantitates the effect. In this instance over a 5 log drop in titers was demonstrable. This observation suggests that the primary infectious unit in Babesia is destroyed by grinding, hence must be relatively large in size.

B. rodhaini - Experiment #4

Object: To evaluate different diluents for use in storing B. rodhaini.

Method: Initially a 1:100 dilution of mouse blood showing a 40-60% parasitemia was prepared using 0.01 M PBS. Using this material a 1:1000 dilution was made by adding 2 ml to 18 ml of the following preparations.

1.) 6% DMSO in 0.01 M PBS pH 7.2

2.) 0.01 M PBS pH 7.2

3.) 0.01 M PBS to which 10% Normal Bovine Serum was added.

4.) 0.01 M PBS mixed with equal parts of glycerol.

5.) 0.01 M PBS containing 50% glucose.

These 1:1000 dilutions were checked for infectivity by inoculating 2 mice with each of the 5 different preparations. All solutions were then frozen at -65°C, and checked for infectivity 72 hours later.

Results:

	Infectivity before Freezing	Infectivity after Freezing 72 Hours
1.) 6% DMSO	2/2	0/2
2.) PBS	2/2	0/2
3.) PBS - 10% N.B. Serum	0/2	0/2
4.) 50% glycerin in PBS	1/2	0/2
5.) 50% glucose in PBS	2/2	0/2

Conclusions: Infectivity was destroyed by freezing in all diluents. Glycerin and normal bovine serum appeared to be detrimental even before freezing. It may be necessary to control the drop of temperature as described by Barnett for Cattle Babesias, or possibly to go to a lower storage temperature such as liquid Nitrogen.

B. rodhaini - Experiment #5 - Pathogenesis Study

Object: To evaluate the infection produced by B. rodhaini with special reference to PCV, parasitemia, body weight and spleen size. Attempts will be made to determine if there is any correlation between spleen size and parasitemia, PCV and spleen size, total body wieght and spleen size and to evaluate statistical differences between normal and infected mice.

Method: A total of 40 mice were used. Twenty were infected by inoculating I.P. 0.5 ml 10^{-3} dilution of B. rodhaini infected blood having a demonstrable parasitemia of over 20%. Twenty mice served as non-infected controls. Seven days later all mice were anesthetized using chloroform. A cardiac puncture was made using 1 ml tuberculin syringe. A maximum amount of blood was removed from each animal and recorded individually. A hematocrit, heparinized,

capillary tube was filled from the syringe for each mouse and identified. A blood smear was made, fixed immediately in absolute methanol, and later stained with Giemsa to determine parasitemia. The mouse was then euthanized and the spleen removed and placed in pre-weighed containers. A total body weight was determined and the mouse discarded. Spleen weight, parasitemia, and PCV values were determined in each animal.

The above values were tabulated, and statical evaluation of all items made to determine differences between infected and normal mice. Correlation-Regression analysis was done on those items indicated to determine possible relationship between various factors in the infected mice.

Results: Comparisons between normal and infected mice are presented in Table VI. The average PCV of infected mice was significantly lower and the average spleen weights were significantly higher. No significant difference in body weight or volume of blood removed by cardiac puncture could be detected. Although not recorded a high percentage of the infected mice showed a hemoglobinuria.

The regression analysis failed to show any significant correlation between PCV and spleen size. A negative correlation between spleen size and parasitemia exists with a probability of error < 0.05. The higher the parasitemia the smaller the spleen size. Among the normal mice no correlation between total body weight and spleen size could be demonstrated.

TABLE VI

Effects of *Babesia rodhaini* Infection in Mice.

	Number of Animals	Average PCV	Average Body Wt.	Average Spleen Size	Average Percent Parasitemia	Blood Volume Removed by Cardiac Puncture
Normal mice	20	44.7	30.5	176.35 ±36.7	0	0.57 ml
Infected mice	20	19.2	31.59	500.55 ±127.38	72.4%	0.48 ml
Significance	-	P<0.01	N.S.	P<0.01	-	N.S.

Conclusions: The effects of B. rodhaini are quite similar to those of Babesia in other animals. There appears to be a primary hemolytic anemia, sufficiently acute to produce hemoglobinuria. Splenomegalia is pronounced, which goes along with other Babesia infections. The relationship between parasitemia and spleen size probably reflects correlation between spleen size and the stage of infection, and the parasitemia correlation is secondary. Splenomegalia is probably greatest in the early stages of infection before the higher parasitemias occur, hence the negative correlation. This point is not proven in these trials however.

B. rodhaini Experiment #6 - Treatment

Object: To compare the efficacy of ganaseg and Burroughs Wellcome 4A65 in treating Babesia infected mice.

Method: A total of 10 mice were used, 4 were untreated controls and 6 were treated. Three were treated with ganaseg, and 3 with 4A65. The 3 treatments were given 24, 48, and 72 hours after exposure. All 10 mice were infected on day 0 by injecting I.P. 0.5 ml 10^{-2} dilution of infected blood showing an 11% parasitemia. Ganaseg and 4A65 were injected at the rate of 2 mg/kg bw I.P. No parasitemia determinations were made on the controls, but parasitemias were made on all treated animals at various intervals.

Results: The individual results are tabulated in Table VII. When treatment was delayed 72 hours both mice died of acute babesiosis. Treatment at 24 and 48 hours successfully prevented death in both groups, but parasitemias developed more markedly in the ganaseg group.

Conclusions: At the dose level of 2 mg/kg the 4A65 was obviously more effective in delaying acute infection and suppressing the Babesia parasitemia.

TABLE VII

Treatment of *Babesia* Infected Mice with Ganaseg and 4A65.

Controls	Time of Treatment	1 0/4	3 1/4	5 1/4	6 4/4	7 X	8 X	11 X	14 X
Ganaseg	24 hours	0	3%	13%	42%	NT	36%	0	0
4A65	24 hours	0	1%	0	0	NT	0	0	31%
Ganaseg	48 hours	0	1%	17%	43%	NT	35%	0	10%
4A65	48 hours	0	27%	0.3%	0		0	0	40%
Ganaseg	72 hours	0	6%	61	65	X	X	X	X
4A65	72 hours	0	3%	88	92	X	X	X	X

B. rodhaini Experiment #7 - Treatment and Immunity

It is apparent that 4A65 is very effective against *B. rodhaini*. The purpose of this experiment was to determine the comparative value of repeated treatment as opposed to a single treatment. A secondary objective was to determine if treated and recovered mice are resistant to challenge.

Method: A total of 15 mice were inoculated I.P. with 0.5 ml 10^{-2} dilution of infected blood. Three mice were not treated and served as controls. The experimental drug 4A65 was used for treatment and was administered at the rate of 2 mg/kg bw I.P. Two mice received a single treatment the day of infection (day 0); 2 mice were treated on day 0 and then on days, 5,7, and 9; 2 mice received a single treatment on day 1; 2 were treated on day 1, 5, 7, and 9; 2 received a single treatment on day 2; 2 were treated days 2, 5, 7, and 9. All surviving mice were challenged 42 days after infection by injecting 0.5 ml of a 1:100 dilution of infected blood in PBS.

Results: The results of treatment and challenge are given in Table VIII. In 2 instances the single treatment while retarding death did not prevent it. A single treatment at 48 hours prevented death but not a parasitemia. Multiple treatments prevent death in every instance. Of the 8 surviving animals 2 died following challenge, but 1 death was accidental and not due to *Babesia*.

Conclusion: Multiple treatments were successful in preventing death loss from *Babesia*, and in one instance a single treatment was successful. The

reason for the multiple treatment was to remove entirely the Babesia infection. In anaplasmosis once an animal is free of infection it becomes fully susceptible to challenge. In this instance considerable protection to challenge was noted in that 6 of 8 survived. The question now arises whether or not these mice were carriers even after the 4 treatments or if they are resistant to challenge due to a sterile immunity.

TABLE VIII

The Effects of Treatment with 4A65 on *Babesia* Infected Mice.

Group	No. of Animals	Days on which Treatment was given*	No. Showing Parasitemia	No. of Deaths	Days Deaths Occurred	No. Challenge	Time of Challenge	No. Dying from Challenge
1	3	No Treatment	3	3	9	-		-
2	2	0	2	2	28	-		-
3	2	0,5,7,9	0	0	-	2	42 days	1**
4	2	1	2	2	28	-		-
5	2	1,5,7,9	0	0	-	2	42 days	0
6	2	2	2	0	-	2	42 days	1
7	2	2,5,7,9	2	0	-	2	42 days	0

* Day 0 - The day of infection.
** - Not due to *Babesia*.

B. rodhaini Experiment #8 - Nature of Immunity

Object: The question of a sterile immunity was raised in experiment #7, and it seemed desirable to study the immune state more thoroughly, with special consideration being given to whether or not infection could be transferred from immune mice.

Method: A total of 16 mice survived B. rodhaini infection following treatment with 4A65. These mice were challenged September 12, October 3, and November 19 with 0.5 ml 10^{-3} dilution of Babesia infected blood. All 16 animals were apparently solidly immune. Each mouse was anesthetized and 0.2 ml blood withdrawn by cardiac puncture, in a heparinized syringe. Immediately 0.1 ml of undiluted blood was inoculated into each of 2 mice. The donner mouse was put into the pen with the two recipients for purpose of identification. Three of the donner mice died overnight apparently due either to the anesthesia or cardiac puncture.

Results: Babesia parasitemia occurred in 4 of 16 passages. In every instance both recipient mice died. Deaths occurred on day 9, 9, 11, and 14. It was concluded that 4 of 16 mice so examined were carriers of the infection, which would explain their resistance to challenge. The other 12 mice must at this time be considered as having a sterile immunity.

Conclusion: Sterile immunity has been reported previously for Babesia, but such an immunity does deviate from the normal pattern of premunition in most hemotropic diseases. Plans are underway to splenectomize the 12 mice from which transmission was impossible. Daily parasitemias will be made following this treatment. If a sterile immunity is a reality then future studies will include duration of immunity.

Babesia rodhaini Experiment #9 - Adjuvant Vaccine

It is desirable to determine the immunizing potential of *Babesia* antigen as a basis for future studies in the area of killed vaccines. If immunity is possible with killed *Babesia* antigens a basis for cross immunological tests may result which would contribute to our understanding of the relationship of 1 *Babesia* to another. Such studies of a basic nature would be in addition to the obvious significance in possible future control programs.

Method: A maximum quantity of blood was collected from 20 mice showing heavy *Babesia* parasitemias (average 72.4%). After the removal of serum the red cell clot was homogenized in a Vir-Tis tissue grinder and centrifuged 45 minutes at over 35,000 XG at 5°C. The sediment was collected, washed 1 time in 0.025% NaCl and collected once again at the same centrifuge setting. This sediment weighing 1.0645 grams was mixed with 52.2 ml V.B. pH 7.4 making a 2% suspension and ground in the Vir-Tis. This antigen was frozen at -20°C until used in the preparation of a vaccine. Vaccine preparation consisted of:

Antigen base (2%)	50%
M.O. + 2 mg/ml M.B.	40%
Arlacel A	10%

The above material was homogenized in a Vir-Tis by mixing at low speed. The resulting vaccine was used as follows:

1.) 5 mice were injected with 0.1 ml vaccine I.P. - 11/26 and 12/29

2.) 5 mice were injected with 0.1 ml vaccine I.P. - 11/26 only

3.) 5 mice were injected with 0.1 ml vaccine subcutaneously - 11/26 and 11/29

4.) 5 mice were injected with 0.1 ml vaccine S.C. - 11/26 only.

A challenge using a 10^{-3} dilution of infected blood was administered January 15, 1970, to each of 20 mice in the above 4 groups plus a group of 5 non-vaccinated mice as controls.

Results: As of this writing results are not complete.

Theileria Experiments

The antigenic and serologic relationship between *Theileria cervi* and the cattle *Theileria* has not been established but the morphologic, epizootiologic and pathogenic similarties are striking, and cannot be disregarded. For this reason it is thought that *T. cervi* can serve as a study model, that might provide useful information applicable to the more serious problems of *T. parva*, and *T. annulata*.

Theileria Experiment #1 - CF Antigen Preparation

Splenectomized deer 165 - showing a 44% *Theileria* parasitemia with a PCV of 15% served as a donner for 300 ml of blood, used in the preparation of a CF antigen. The erythrocytes were washed 3 times in Veronal Buffer. Packed erythrocytes were frozen at -20°C. Several days later the frozen cells were thawed and centrifuged 35,000 XG for 1 hour. The sediment was mixed with cold distilled water in a Vir-Tis for 15 seconds. The heavy foam which developed during grinding was broken and dispersed with Octanol. The suspension was centrifuged 1 hour at 35,000 XG. The sediment was collected, resuspended in distilled water and centrifuged, twice more to remove hemoglobin. A final sediment was prepared as a 2% suspension in V.B. and tested for evidence of complement-fixing activity against known positive and negative serum.

Results: The following tabulates the antigen titration which indicates specific complement-fixing activity.

Tube	1	2	3	4	5	6	7	8	9
1:5 Antigen Amt.	0.05	0.1	0.15	0.20	0.25	0.30	0.35	0.40	0.45
2 units complement + .5 ml of a 1/5 dilution of serum + Hemolytic System									
Pos. Ser.	1+	2+	3+	3.5+	4+	4+	4+	4+	4+
Neg. Ser.	-	-	-	-	-	-	Tr.	Tr.	Tr.

Conclusions: A trace of anticomplementary activity was noted in the higher antigen amounts, but specific activity occurred below these amounts.

If 1 unit = .25 of a 1:5 then 2 units = .5 of a 1:5 dilution. The titer is low but specific and could be of use as a serologic test.

Theileria Experiment #2 - Cross reactions

One attempt to demonstrate a serologic relationship between Anaplasma and Theileria was unsuccessful. The approach used consisted of antigen titrations against positive, and negative serums from Theileria positive and negative deer, and Anaplasma positive and negative bovine. By these means no evidence of cross reactions were detected. Further studies are indicated, however and will be made. It is not uncommon for reactions to occur to the Anaplasma CF test with serum from deer showing a Theileria parasitemia. This reaction has been observed mainly in those splenectomized deer where the parasitemia is undergoing a Theileria influx, and then the CF reaction recedes with the diminishing parasitemia. This observation may of course be due to a non-specific factor, but will be investigated more thoroughly in the future.

Miscellaneous Experiments

Plans have been made to include total erythrocyte (RBC) and leucocyte (WBC) counts in the evaluation of future trials. To do this an electronic counter manufactured by Fisher Laboratories designated an "Autocytometer" has been purchased.

Prior to putting the machine into routine use a series of familiarization trials have been conducted to establish approximate parameters of dependability. Repeated counts have been made on the same samples to arrive at the standard deviation of RBC, WBC, and PCV values of oxalated blood samples at varying times. In addition comparisons have been made between manual and autocytometer counts. A Coulter Counter was used as a check in 1 series of trials to establish relative agreement between 2 electronic counters of different manufacture, and the relative standard deviations of repeated counts on the same sample by the 2 machines.

A trial has been set up to ascertain the relative reliability of counts on RBC to which 0.2% or 10% formalin has been added.

Trial 1 A quantity of blood was collected in balanced oxalate crystals from calves 136, 190, 191 and 419. Six coded tubes were filled from each animal. Packed cell volumes (PCV), RBC, and WBC were made on each of the 24 blood tubes (4 X 6) at 0-2 hrs, 6 hrs, 24 hrs, 48 hrs, 5 days and 7 days. All samples were refrigerated between counts and thoroughly mixed before counts were made.

Standard deviations were done for each of the 6 tubes on each animal at all countings. At the conclusion an analysis of variance was conducted to determine if counts on each animal were significantly different as the samples became increasingly older for each of the 3 factors (PCV, RBC, WBC).

Table XI tabulates the results for this trial, and is divided in 3 parts one for each of the 3 types of evaluation PCV, RBC, and WBC.

Time is a factor in RBC. No significant difference was detected in counts taken 0-2, 6, and 24 hours. Counts at 5 days were lower in 2 of the 4 and lower in 3 of 4 at 7 days. An instrument error was detected at 48 hours with all 4 samples reading high. Standard deviations were within 0.5 million, which is considered satisfactory duplication.

WBC counts appeared to have been high at 48 hours. Experience has shown that a slightly dirty counting chamber can significantly influence counts, and it is supposed that this may have occurred. No consistant pattern of differences in total WBC, relative to time, emerges as a result of this study. Standard deviations are somewhat higher at 48 hrs,--5 and 7 days. The standard deviation for WBC are proportionately larger than seen with RBC or PCV values.

Significant differences in PCV values for the various times tested were detected in 3 of the 4 animals tested. In one animal, 191, this difference was minimal. In all 3 the significantly different value occurred at 5 and 7 days. No significant differences were apparent at 0-2 hrs, 6 hrs, 24 and 48 hrs determination. Standard deviations were relatively lower than for either the WBC or RBC determinations.

Conclusion: Using oxalate as anticoagulant blood samples may be counted by these techniques up to 24 hrs after collection and probably 48 hrs without significantly influencing the results.

TABLE XI

Average PCV Values on 4 Calves Over a 7 Day Period.

Animals:		136	190	191	419	Average S.D. for each count
Values at:	0 hrs.	28.2 ± .8	28.3 ± .4	12.0 ± 0	24.5 ± 1.0	.32
	6 hrs.	28.2 ± .45	29.0 ± 0	12.0 ± 0	25.7 ± 1.6	.51
	24 hrs.	28.8 ± .4	28.8 ± .4	11.8 ± .4	24.8 ± .4	.40
	28 hrs.	28.8 ± .4	29.2 ± .4	12.0 ± 0	24.8 ± .4	.30
	5 days	27.8 ± .4	30.0 ± 0	11.5 ± .5	24.8 ± .4	.32
	7 days	29.2 ± .8	30.3 ± .5	12.2 ± .4	25.5 ± .5	.55
Avg. S.D. for each animal		± 0.54	± 0.28	± 0.22	± 0.72	
Significance		P<0.01	P<0.01	P<0.05	NS	
DRS*		0.96	0.98	0.57	--	

*DRS: Difference required for significance.

SD: Standard Deviation.

TABLE XI

Average RBC Values on 4 Calves over a 7 Day Period.

		RBC (Millions)				
Animals:		136	190	191	419	Average S.D. for each count
Values at:	0 hrs	9.9 ± 1.1	8.6 ± .4	3.4 ± .1	8.2 ± .2	.45
	6 hrs	9.2 ± 0.5	8.5 ± .24	3.4 ± .22	8.3 ± .24	.30
	24 hrs	9.1 ± .4	8.4 ± .2	3.4 ± .1	8.4 ± .1	.20
	48 hrs	9.6 ± .4	9.2 ± .5	4.0 ± .5	8.8 ± .3	.42
	5 days	8.1 ± .2	8.1 ± .3	3.7 ± .5	7.6 ± .4	.35
	7 days	8.1 ± .7	7.4 ± .3	3.2 ± .1	7.2 ± .3	.35
Avg. S.D. for each animal		0.55	.32	.25	.26	
Significance		P<0.01	P<0.01	P<0.01	P<0.01	
DRS		1.1	0.56	0.54	0.47	

DRS: Difference required for significance.

SD: Standard Deviation.

TABLE XI (continued)

Average WBC Values on 4 Calves over a 7 Day Period.

		WBC (Thousands)				
Animals:		136	190	191	419	Average S.D. for each count
Values at:	0 hrs.	9.4 ± .6	9.5 ± .7	15.0 ± .8	8.8 ± .5	0.65
	6 hrs.	11.9 ± .6	11.9 ± .7	17.1 ± .5	11.7 ± .6	0.60
	24 hrs.	11.0 ± .7	10.8 ± .4	16.0 ± .8	10.2 ± .4	0.58
	48 hrs.	12.3 ± 1.7	12.9 ± 1.7	16.5 ± .7	11.4 ± 1.2	1.32
	5 days	9.9 ± .2	9.9 ± .5	15.5 ± 1.7	10.8 ± .7	0.78
	7 days	8.9 ± .4	7.7 ± .6	14.5 ± 1.3	10.1 ± .5	0.70
Avg. S.D. for each animal		.70	.77	0.97	0.65	
Significance		$P<0.01$	$P<0.01$	$P<0.01$	$P<0.01$	
DRS		1.47	1.5	1.8	1.3	

DRS: Difference required for significance.

SD: Standard Deviation.

Trial #2 A second series of trials were designed to compare WBC obtained by manual counts and counts obtained on the Autocytometer. Considerable improvement in counting technique, especially with the Autocytometer, has been noted. For this reason that portion of trial 1, dealing with WBC was repeated. Previously no clear pattern of storage effects on WBC was detected (Trial 1).

A total of 5 replicate samples from 4 calves (138, 188, 422 and 500) were given coded numbers ranging from 1-to-20 and were manually counted at 1-4 hours, 24 hours, and at 48 hours. WBC were determined on the same samples 1-4 hours, 24 hours, 48 hours, 5-7 and 9 days.

Standard deviations (S.D.) were calculated on each set of replicates. Manual and Autocytometer S.D. values were compared statistically. WBC values at 1-4 hours, 24 hours, 48 hours, 5-7 and 9 days were evaluated to determine the presence of statistical differences.

Results are tabulated in Tables XII and XIV. The S.D. of manual counts were over 6 times higher than those obtained using the Autocytometer. This difference is highly significant.

When counts are made on the Autocytometer no differences were apparent in counts made up to 24 hours after collection. Two of 4 showed significantly lower WBC at 48 hours and 1 of 4 was lower at 5 days. WBC were significantly lower in all animals at 7 and 9 days after blood was collected.

Conclusion: Significant but small drops in WBC might be expected in blood samples stored 48 hours or more. The drop after 5 days becomes consistant and of greater magnitude.

TABLE XII

Total WBC as Determined Manually and on the Autocytometer (Auto).

		138	188	422	500	Avg. S.D. Manual	Avg. S.D. Auto
<4 hrs.	Manual:	7.99 ± 2.4	15.16 ± 6.8	7.7 ± 4.5	6.43 ± 0.8	3.62	
	Auto:	8.12 ± 0.4	17.42 ± 1.2	8.08 ± 0.4	6.88 ± 0.2		.55
24 hrs.	Manual	8.73 ± .85	20.06 ± 1.3	8.0 ± .52	7.13 ± 2.34	1.25	
	Auto	8.39 ± .62	17.72 ± .23	8.05 ± .30	6.83 ± .21		.34
48 hrs.	Manual	6.32 ± 2.54	19.96 ± 3.92	6.35 ± 2.6	5.06 ± .76	2.46	
	Auto	7.16 ± 29	17.04 ± .26	7.2 ± .29	5.94 ± 24		.27
Avg. S. D.						2.44	0.39

SD: Standard Deviation.

TABLE XIV

Total WBC as Determined on Autocytometer from 0 to 9 Days.

	138	188	422	500	Avg. S. D.
<4 hrs.	8.12 ± .4	17.42 ± 1.2	8.08 ± .4	6.88 ± .2	0.55
24 hrs.	8.39 ± .62	17.72 ± .23	8.05 ± .3	6.83 ± .21	0.34
48 hrs.	7.16 ± .29	17.04 ± .26	7.2 ± .29	5.94 ± .24	0.27
5 days	6.35 ± .49	17.84 ± .25	6.81 ± .33	6.44 ± .23	0.32
7 days	4.56 ± .27	15.64 ± .30	5.25 ± .34	4.67 ± .40	0.33
9 days	5.17 ± .45	14.7 ± .28	5.7 ± .78	5.47 ± .41	0.48
Significance	P<0.01	P<0.01	P<0.01	P<0.01	
DRS	0.84	1.03	1.9	0.55	

DRS: Difference required for significance.

SD: Standard Deviation.

Trial #3 A Coulter cell counter is in operation in the Clinical Pathology Laboratory of the Veterinary Clinic. It was decided that comparisons of counts obtained from the Coulter and the Autocytometer would be useful in more fully evaluating the latter.

Essentially the same technique was followed as described in trial #2. A total of 5 replicate samples from 4 calves (138, 188, 422, 500) were given coded numbers ranging from 1 to 20 and were counted the same day as collected on the Coulter Counter and the Autocytometer. The counts on the Coulter were done by the technician who normally operates this machine, and those on the Autocytometer were done by our technician who is familiar with this equipment. Both RBC and WBC were determined. Standard deviations were determined and compared statistically. Average counts were also compared and evaluated statistically.

The standard deviations were comparable in both RBC and WBC with no significant differences occurring. RBCs on the Coulter and Autocytometer failed to reveal any significant differences. The Coulter Counter gave a significantly higher WBC on calf 188, but all other counts were essentially the same, no significance being noted.

Conclusion: The Autocytometer eventhough less than 1/2 the price of a Coulter Counter gave comparable WBC and RBC in bovine blood. The standard deviation as determined on replicates was essentially the same.

TABLE XIV

WBC and RBC Made on Coulter Counter and Autocytometer.

	138	188	422	500	Average S.D. Coulter	Average S.D. Auto
RBC: Coulter	7.74 ± .63	4.01 ± .31	7.92 ± 1.0	7.18 ± .44	0.595	
Auto	8.6 ± .55	4.00 ± .50	7.38 ± .63	6.88 ± .47		0.538
Significance	NS	NS	NS	NS	NS	
WBC: Coulter	7.94 ± .41	20.52 ± .91	7.45 ± .39	7.02 ± .24	0.49	
Auto	7.39 ± .36	18.6 ± .62	7.34 ± .47	6.78 ± .42		0.48
Significance	NS	$P < 0.01$	NS	NS	NS	

SD: Standard Deviation.

NS: Not Significant.

Future Work

Major emphasis will continue in the area of anaplasmosis in the primary areas of:

1.) Development of practical treatment program for eliminating anaplasmosis infection.

2.) Development of a safe and reliable method of premunization using virulent A. marginale.

3.) Production of a killed vaccine, eliminating bovine erythrocytic antigens which are believed to be responsible for iso-erythrolysis of baby calves.

4.) Environmental factors responsible for alterations in virulence will be further investigated if facilities or circumstances allow.

Babesia:

1.) An experiment is currently underway to determine if Boophilus annulatus can become a vector for Babesia cervi in deer. It will also be determined if Babesia cervi can be transferred to cattle via the Boophilus tick. (Nuevo Laredo)

2.) Babesia transmission studies currently reported on B. annulatus will be repeated using B. microplus (Nuevo Laredo).

3.) A killed adjuvant vaccine will be prepared and tested using B. cervi in deer.

Babesia rodhaini :

1.) Basic studies on the immunologic aspects of B. rodhaini will continue with special attention being given to sterile immunity.

2.) Attempts will be made to adapt B. rodhaini to a larger rodent specie for future serologic studies.

3.) Serologic studies will be made to characterize possible common antigens between the different Babesia organisms. (rodhaini and cervi.)

4.) Attempts will be made to develop a technique of neutralization tests for B. rodhaini.

Theileria

Previous work has indicated an immunological relationship between Theileria cervi and Anaplasma marginale . Preliminary studies not reported here have failed to repeat this finding. This work will be completed this year, but will require a fairly large number of deer, and efforts to obtain these are now underway.

A serologic relationship to Anaplasma has been noted, but efforts reported this year have not succeeded in fully characterizing this relationship. Further efforts will be made along this line.

Student Training Activities at Texas A&M

Dr. C. A. Carson, Texas A&M University, successfully completed work for a Master of Science Degree in Microbiology. His thesis was entitled: "An Antigenic and Serologic Comparison of Two Virulent Strains and an Attenuated Strain of *Anaplasma marginale*." A copy of the abstract is attached to this report.

Dr. George L. Corry, EAVRO - Muguga Kenya, successfully completed work for a Master of Science Degree in Microbiology. Dr. Corry was financed in this effort by a stipend from FAO. His thesis was entitled: "Preparation of a Soluble Antigen from *Theileria cervi* and its Application to Various Serologic Tests." A copy of the abstract is attached to this report.

Drs. Kenneth C. Thompson (Microbiology) and Rollie G. Corrier (Pathology) are currently enrolled in graduate school, Texas A&M, and will be completing course work in June, 1970 for the Master of Science degree.

Dr. John P. Bishop (Microbiology) is currently enrolled in graduate school, Texas A&M, and will be completing course work for the Ph.D. degree probably in June, 1970.

Drs. Floyd M. Jones (Microbiology) and Charles A. Daley (Pathology) successfully completed their course work at Texas A&M June, 1969, and are now in Colombia completing work for the Masters degree.

Dr. Hernan Zaraza, Colombia, has been working with the Texas A&M staff since last September, and pursuing course work in English in preparation to enter graduate school February, 1970. Dr. Zaraza has taken the GRE, but the test results have been delayed, so full admission to graduate school has been delayed. A favorable recommendation has been received from the guidance and counseling service, Texas A&M, with regards to Dr. Zaraza's competency in English. Dr. Zaraza can begin his course work this February if the Rockefeller scholarship is approved as a special student.

ABSTRACT

AN ANTIGENIC AND SEROLOGIC COMPARISON OF TWO VIRULENT STRAINS AND AN ATTENUATED STRAIN OF *ANAPLASMA MARGINALE*

Charles Andrew Carson

V.M.D., University of Pennsylvania

Directed by: Drs. L. C. Grumbles and K. L. Kuttler

An antigenic and serologic study was conducted using virulent strains of *Anaplasma marginale* from Texas and Colombia and an attenuated strain of *Anaplasma marginale*. Soluble antigens of the three *A. marginale* strains were compared by agar gel diffusion and immunoelectrophoresis. Serum proteins from calves infected with each of the three *A. marginale* strains were separated electrophoretically and reacted with rabbit anti-bovine serum in immunoelectrophoresis systems.

No differences between the soluble antigens of the three *A. marginale* isolates were detectable by agar gel diffusion. All three antigens moved to the same mobility zone in agar gel electrophoresis systems and each antigen formed an arc of precipitation when reacted with serum from calves infected with homologous or heterologous strains of *A. marginale*.

A *beta* and a *gamma* serum protein component, not exhibited in normal bovine serum, were present in the serums of animals infected with either of the virulent *A. marginale* strains or the attenuated strain.

ABSTRACT

Preparation of a Soluble Antigen from *Theileria cervi* and its Application to Various Serologic Tests. (January 1970)

George L. Corry, B.V.Sc., University of East Africa;

Directed by: Dr. [illegible] L. Kuttler

Theileria cervi serologic antigen was obtained from the erythrocytes of a deer infected with *T. cervi*. The animal was bled by venipuncture, when 20 per cent of its erythrocytes were parasitized. The blood was hemolyzed by freezing at -20°C. A second blood sample was hemolyzed by adding distilled water. The two antigens obtained were solubilized by sonication, titrated by complement-fixation (CF) reaction, pooled and used in CF, hemagglutination (HA) and precipitin tests.

The solubility of the antigen in buffer solvents as determined by CF reaction was in direct proportion to the rise in pH. It was highest at pH 11. For the CF and HA tests however the pH 10 buffer was used as solvent.

Thirty-four serums obtained from six deer infected with *T. cervi* were used in the CF and HA tests. Both tests detected the presence of antibody to the parasite.

Two precipitin tests; the ring precipitin and the agar gel diffusion tests gave no valid results.

Publications - 1969

Kuttler, K. L., Adams, L. G., and Zaraza, H.: An Epizootiologic and Geographic Survey of Anaplasma marginale and Trypanosoma theileri in Colombia. Presented at the AVMA Convention, July, 1969, Minneapolis, Minn., J.A.V.M.A. 154 (June 1, 1969) p. 1398. Also submitted for publication to Revista I.C.A.

Gadir, F. A., Hidalgo, R. J., and Kuttler, K. L.: Complement-Fixation Antigen Production for Theileria in White Tailed Deer (Odocoileus virginianus). Submitted to Am. J. Vet. Res.

Kuttler, K. L. and Zaraza, H.: A Preliminary Evaluation of a Dithiosemicarbazone for the Treatment of Anaplasmosis. Accepted for publication in Res. in Vet. Sci.

Kuttler, K. L. and Adams, L. G.: Comparative Efficacy of Oxytetracycline and a Dithiosemicarbazone in Eliminating Anaplasma marginale Infection in Splenectomized Calves. Accepted for publication in Res. in Vet. Sci.

Kuttler, K. L.: Serial Passage of an Attenuated Anaplasma marginale in Splenectomized Calves. Presented at the U.S.-A.H.A. meetings, Oct. 13-16, 1969, Milwaukee. Accepted for publication in the U.S.-A.H.A. Proceedings.

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Adams, L. G. and Kuttler, K. L.: Observations on the Toxicity of a-Ethoxyethylglyoxal Dithiosemicarbazone in Cattle. Submitted to Am. J. of Vet. Res.

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Adams, L. G. and Kuttler, K. L.: Observations on the Toxicity of a Ethoxyethylglyoxal Dithiosemicarbazone in Cattle. In press.

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Carson, C. A., Adams, L. G., and Todorovic, R. A.: An Antigenic and Serologic Comparison of Two Virulent Strains and an Attenuated Strain of Anaplasma marginale. Am. J. of Vet. Res., Vol. 31, No. 6, June, 1970, pp. 1071-1078.

Gadir, F. A., Hidalgo, R. J., and Kuttler, K. L.: Complement-Fixation Antigen Production for Theileria in White-Tailed Deer (Odocoileus virginianus). Am. J. Vet. Res., 31, May, 1970, pp. 879-885.

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Kuttler, K. L. and Zaraza, H.: A Preliminary Evaluation of a Dithiosemicarbazone for the Treatment of Anaplasmosis. Accepted for publication in Res. in Vet. Sci.

Kuttler, K. L. and Adams, L. G.: Comparative Efficacy of Oxytetracline and a Dithiosemicarbazone in Eliminating Anaplasma marginale Infection in Splenectomized Calves. Accepted for publication in Res. in Vet. Sci.

Kuttler, K. L.: Serial Passage of an Attenuated Anaplasma marginale in Splenectomized Calves. Presented at the U.S.A.H.A. meetings, Oct. 13-16, 1969, Milwaukee. Accepted for publication in the U.S.A.H.A. Proceedings.

Kuttler, K. L. and Zaraza, H.: Premunization with an Attenuated Anaplasma marginale. Presented at the U.S.A.H.A. meetings, Oct. 13-16, 1969, Milwaukee. Accepted for publication in the U.S.A.H.A. Proceedings.

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Kuttler, K. L., Graham, O. H., and Johnson, S. R.: Boophilus annulatus as a Vector for Anaplasmosis in Cattle and White-Tailed Deer (Oodocoileus virginianus). Submitted for publication.

Todorovic, R. A., Lugue, Gonzalo, and Adams, L. G.: Contribucion al Estudio de la Distribucion de Garrapatas en Colombia, Sur America. Submitted for publication.

Dr. Maurer

NECROBACILOSIS NEO-NATAL EN OVINOS

Adams, L.G., DVM, Ph.D. and Ferreira, W.L., M.V.

INTRODUCCION

La necrobacilosis hepática y pulmonar es una enfermedad necrotizante del hígado y pulmones que se presenta con mayor incidencia en terneros y corderos. En los corderos la enfermedad generalmente ataca animales de menos de 2 semanas de edad y usualmente causa alta mortalidad (15). Mack en 1915 (citado por Marsh (15)) describió un brote en Nevada que mató más de 1500 corderos en una sola estación. La enfermedad en el hígado y en los pulmones de los corderos parece originarse por una infeccion inicial en el cordón umbilical (1, 10, 15, 17). Nieberle (17) llama también la atención sobre la transmisión placentaria al feto. Actualmente se acepta al Spherophorus necrophorus como agente etiológico de la necrobacilosis hepática y pulmonar, y la necrosis de las patas (1, 2, 3, 4, 6, 8, 9, 11, 15, 16, 18, 21, 22, 24).

Este trabajo tiene por finalidad llamar la atención sobre la ocurrencia de la enfermedad en Colombia, presentando su historia, signos clínicos, las lesiones macro y microscópicas y los métodos de prevención y control.

HISTORIA Y SIGNOS CLINICOS

Los corderos tenían historia clínica de haber nacido en

buen estado de salud y sin presentar ninguna anormalidad. Los únicos síntomas observados antes de la muerte fueron ligero meteorismo; dolor abdominal o torácico, evidente a la palpación y percusión; depresión y muerte rápida. Algunos animales murieron subitamente sin presentar ningún síntoma en los primeros 14 días de vida. Por lo menos 10 ovejas presentaron lesiones macroscópicas de necrosis de las patas de las cuales se tomaron muestras con hisopos para cultivo bacteriológico. Aproximadamente 25 ovejas se examinaron clinicamente para buscar evidencia de mastitis gangrenosa, sin resultados positivos, 10 muestras de leche se recolectaron para cultivo bacteriológico.

Durante la noche las ovejas se guardaban en un cobertizo para protegerlas del viento frío de las montañas y de las lluvias, sin embargo, había numerosas goteras en el cobertizo y el piso de tierra presentaba constantemente áreas fangosas. Poer esta razón, los cascos de las ovejas, siempre estaban húmedos, sucios y contaminados. Se recolectaron cuatro muestras de las áreas fangosas para examen bacteriológico. Aproximadamente 20 corderos murieron durante este brote de un grupo de 200 corderos recién nacidos.

LESIONES MACROSCOPICAS

Los cinco animales examinados presentaban regular estado de nutrición, con discreta cianosis de las mucosas explorables.

La piel y el pelaje tenían apariencia normal. El hígado presentaba masas necróticas ovaladas o redondeadas de bordes relativamente definidos, de coloración blanquecina, o blanco-grisáceo, opaco, relativamente salientes y las cuales eran más numerosas en el lóbulo ventral que en el dorsal (Fig. 1). También se observó en uno de los animales examinados un gran absceso, relativamente bien circunscrito por una cápsula delicada, el cual estaba adherido al diafragma y al piso de la cavidad abdominal. Había tambíen masas necróticas voluminosas que ocupaban casi enteramente un lóbulo hepático, circundadas por una fina banda de tejido hepático y de hiperemia.

Los pulmones presentaban numerosas zonas necróticas redondeadas, de coloración blanquecina o blanco-grisácea opaca, de 2-3 cm. de diámetro, especialmente en la pleura visceral, las cuales estaban adheridas al diafragma y a la superficie pleural parietal de la pared de la cavidad torácica (Fig. 2). Las porciones del pulmón menos severamente afectadas estaban hiperémicas y consolidadas en las áreas de declive. Los demás órganos no presentaban lesiones relacionadas con la enfermedad. El material recolectado para estudio histopatológico se colocó en formalina al 10% buferada, para posterior inclusión en parafina.

LESIONES MICROSCOPICAS

El hígado presentaba hiperemia, hemorragia, metamorfosis

grasa y pequeñas áreas de necrosis de coagulación (Fig. 4) que iban hasta extensas áreas de necrosis de caseificación. El parénquima hepático cerca a esas áreas de necrosis presentaba alteraciones regresivas, disociación de las láminas epiteliales hepáticas y dilatación sinusoidal. En la periferia de estas áreas necróticas había una zona de reacción inflamatoria con leucocitos fragmentados y hepatocitos necróticos así como numerosos organismos bacterianos pleomórficos, observados más adentro del área necrótica (Fig. 5, 6). Las areas portales a menudo estaban llenas con linfocitos, tejido de granulación joven e hiperplasia biliar temprana.(Fig. 7, 8). Estos organismos con coloración de Brown-Brenn eran Gram-negativos, pleomórficos con predominancia de formas filamentosas (Fig.9).

Los pulmones presentaban basicamente las mismas lesiones. Se observaron extensas zonas de infarto (necrosis de coagulación) con micro-organismos en su interior. Los organismos mezclados con una discreta zona de reacción inflamatoria leucocitaria, se observaron también en la periferia de la lesion (Fig. 10). Un gran trombo bacteriano causaba los infartos y consecuentemente proporcionaba un ambiente favorable a la proliferación del organismo (Fig. 11). Los alvéolos dentro de la zona necrótica sufrieron necrosis coagulativa y/o caseosa, y muchos estaban llenos de leucocitos necróticos y restos celulares. Con coloración especial de Brown-Brenn, se observaron, organismos Gram-negativos pleomorficos con predominancia de

filamentos.

BACTERIOLOGIA

Dos conejos fueron inoculados con un macerado de muestras de tejidos de focos hepáticos y pulmonares necróticos, recolec- asepticamente. Los conejos desarrollaron abscesos hepáticos y subcutáneos localizados al octavo día post-inoculación. Frotis de los abscesos, coloreados con Gram, revelaron bacilos delgados, pleomórficos, Gram-negativos. Al cultivar el organismo en caldo de cerebro-dextrosa formó un sedimento muy fino. En agar sangre causó una pequeña zona de hemólisis tipo beta alrededor de la colonia. El organismo produjo ácido y gas en glucosa y maltosa y alguna fermentación en galactosa, lactosa, fructosa y manitol. El organismo resultó negativo al Voges-Proskawer y al test del rojo de metilo y no redujo los nitratos, pero produjo indol. Basado en estas características se determinó que el organismo era el S. necrophorus. De 10 hisopos tomados de lesiones de necrosis del pie, 6 resultaron positivos a S. necrophorus; las 10 muestras de leche fueron negativas; las cuatro muestras del piso del cobertizo resultaron positivas.

DISCUSION

De acuerdo con la literatura consultada, es la primera vez que en Colombia se describe la necrobacilosis en corderos recién

nacidos. La historia clínica indicó que los corderos habían nacido saludables y de buen aspecto, presentando algunos días después decaimiento y muerte rápida, o algunas veces, muerte súbita y sin síntomas; las lesiones macro y microscópicas y los cultivos positivos para S. necrophorus nos llevaron al diagnóstico de necrobacilosis, causada por el S. necrophorus. La historia clínica presentada coincide con la observada por otros autores (1, 6, 15). Los hallazgos de necropsia coinciden con los descritos por otros autores (1, 5, 6, 8, 10, 11, 14, 15, 17, 18, 19, 20, 22, 23).

Las lesiones microscópicas son semejantes a las descritas por varios investigadores (8, 10, 11, 15, 17, 20). El aislamiento, morfología y características del organismo aislado de las lesiones, también concuerda con el hallazgo de otros autores (4, 6, 7, 9, 11, 12, 15, 18, 19, 22). En este brote de necrobacillosis neo-natal, se sospecho que el origen de la infección pudo ser por transmisión transplacentaria o infección umbilical con subsecuente metástasis al hígado y los pulmones. No se observaron lesiones en le ombligo, sin embargo, había una predominancia de focos necróticos en el lóbulo ventral del hígado el cual está directamente conectado a las venas umbilicales. Tampoco había lesiones ruminales que explicaran la diseminación por vía de la circulación portal.

Como se dijo anteriormente este es el primer reporte

conocido en Colombia, de necrobacilosis en corderos recién nacidos, lo que debe llamar la atención de los clínicos e investigadores en relación con el mejor manejo de los rebaños ovinos del país.

PREVENCION

La prevención de la necrobacilosis hepática y pulmonar en animales jóvenes está basada simplemente en la higiene. Se deben lavar, limpiar y desinfectar los sitios donde las ovejas van a dar cría. Después del nacimiento se aconseja cortar el cordón umbilical más o menos 3 cm. por debajo del ombligo. Enseguida se sumerge completamente el ombligo en una solución desinfectante. La tintura de yodo es la mejor y más práctica. Con ese procedimiento se espera que la enfermedad se reduzca al mínimo.

RESUMEN

Se diagnosticaron cinco casos de necrobacilosis neonatal en ovinos jóvenes (menos de dos semanas de edad) en la Sabana de Bogotá. Las lesiones macro y microscópicas se describieron y el diagnóstico se confirmo por cultivos bacteriológicos. Este reporte constituye el primer conocimiento de la incidencia de la enfermedad en ovinos recién nacidos en Colombia.

SUMMARY

Five cases of ovine neo-natal necrobacillosis, in the Sabana of Bogotá, were diagnosed in lambs less than 2 weeks of age. Macroscopic and microscopic lesions were described and the diagnosis was confirmed by bacteriological cultural techniques. This report constitutes the first known incidence of disease in neo-natal lambs in Colombia.

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An Antigenic and Serologic Comparison of Two Virulent Strains and an Attenuated Strain of *Anaplasma marginale*

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SUMMARY

Soluble antigens of 3 *Anaplasma marginale* strains were compared by agar gel diffusion and immunoelectrophoretic techniques. Serum proteins from calves infected with each of the 3 *A. marginale* strains were separated electrophoretically and tested with rabbit anti-bovine serum in immunoelectrophoretic systems. There was no detectable difference between the soluble antigens of the 3 *A. marginale* strains. A *beta* globulin arc, which was not detectable in normal bovine serum, was present in serum of acutely affected calves, and a *gamma* globulin arc was lengthened in the latter serum as compared with that in serum of normal calves.

Various isolates of *A. marginale* in the United States were reported[5] to have antigenic differences. Since an isolate of United States origin was used in the production of a modified live anaplasma vaccine,[14] the present study was made to determine the antigenic compositions of the attenuated organism and a strain from Colombia where the efficacy of the vaccine was being determined by field studies.[7] The antigenic composition of the attenuated organism was also compared with the Texas strain of *A. marginale*. Both the Texas and Colombian strains of *A. marginale* produced a more severe form of the disease than was produced by the attenuated *A. marginale*.

Various soluble antigens have been extracted from hemoparasites, and sensitive methods for detecting their presence and the presence of their antibodies have been described.[1,11,12] One soluble antigen,[1] the exact nature of which was not determined, was used in the present experiment. Since the use of only one

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TABLE 1—Identification of Experimental Calves

Animals	Strain of *A. marginale* in inoculum	Use of animals in the experiment
EXPERIMENTAL CALVES		
1 (splenectomized)	Attenuated (U.S. vaccinal strain)	Produce soluble antigen I*; donor for calves 4 through 7
2 (splenectomized)	Colombian	Produce soluble antigen II*; donor for calves 8 through 11
3 (splenectomized)	Texas	Produce soluble antigen III*; donor for calves 12 through 15
4, 5, 6, 7	Attenuated (U.S. vaccinal strain)	Examine seroimmunologic response
8, 9, 10, 11	Colombian	Examine seroimmunologic response
12, 13, 14, 15	Texas	Examine seroimmunologic response
16, 17, 18, 19 (Controls)	None (saline solution)	Examine seroimmunologic response
OTHERS		
38 (non splenectomized)	None	Produce normal soluble antigen (soluble antigen IV)
700 (splenectomized steer)	Colombian	Donor for calf 2
701 (splenectomized steer)	Texas	Donor for calf 3

* Percentage parasitemia of blood from calves 1, 2, and 3 used for preparation of the 3 soluble antigens was 8, 15, and 12%, respectively.

antigen extraction process does not allow for reporting on complete antigenic characterization of *A. marginale*, the changes in the serum protein of animals infected by the 3 strains were determined to obtain additional information.

Materials and Methods

Nineteen male Holstein-Friesian calves (No. 1–19) were obtained from a commercial dairy on the Bogota Savannah (plateau) of Colombia (Table 1). Each calf was placed in an individual stall and was fed milk and starter rations until approximately 3 months of age. Calves were free of anaplasmosis as determined by results of examinations of Giemsa stained blood smears and of complement fixation (CF) tests done each week for 2 months before they were inoculated. Calves 1, 2, and 3 were splenectomized 20 days before they were inoculated with the strains of *A. marginale*. Calf 1 was used for the production of soluble antigens from the attenuated *A. marginale* (soluble antigen I). Calf 2 was used for the production of soluble antigens from the Colombian strain of *A. marginale* (soluble antigen II). Calf 3 was used for the production of soluble antigens from the Texas strain of *A. marginale* (soluble antigen III). Soluble antigen IV was prepared from red blood cells (RBC) of a normal calf.

TABLE 2—Development of Detectable Antibody to the Soluble Antigen of 3 Strains of *Anaplasma marginale*

Inoculum	Calf No.	Antibody first detected (days)
Soluble antigen I (attenuated vaccinal strain)	4	23
	5	23
	6	23
	7	23
Mean (± S.D.)		23.0 ± 0
Soluble antigen II (Colombian strain)	8	15
	9	36
	10	23
	11	29
Mean (± S.D.)		25.8 ± 15.4
Soluble antigen III (Texas strain)	12	23
	13	19
	14	29
	15	48
Mean (± S.D.)		29.8 ± 22.2

There is no significant difference in the number of days which elapsed prior to detection of the antibody in the 3 groups. ($P < 0.05$.)
S.D. = Standard deviation.

Calves 4 through 19, allotted to 4 groups of 4 animals each, were used to determine the serologic response to *A. marginale* infection. Calves 4 through 7 were infected with the attenuated vaccine *A. marginale*. Calves 8 through 11 were infected with the Colombian strain of *A. marginale*. Calves 12 through 15 were infected with the Texas strain of *A. marginale*. Calves 16 through 19 were used as control animals.

SOLUBLE ANTIGENS OF A. MARGINALE

Inoculation of Calves and Collection of Samples.—Calf 1 was inoculated subcutaneously with 17 ml. and intravenously

with 3 ml. of viable attenuated *A. marginale* in sheep blood. The inoculum had been hand-carried from Mexico[a] to Colombia in a chest (solid carbon dioxide was used as refrigerant) and kept at −65 C. for 6 months prior to use. After calf 1 was inoculated, paired blood samples were collected 3 times each week, with and without added anticoagulant (balanced oxylate).[b] Giemsa-stained blood smears and specimens tested for packed cell volume (PCV) were prepared from blood samples which contained anticoagulant. Serum collected from the clotted samples was tested for CF activity. At the time calf 1 had maximal parasitemia (8%), 500 ml. of blood was collected in a flask containing 25 ml. of 12% sodium citrate solution. The blood was immediately centrifuged[b] in a 4 C. atmosphere at 480 g[c] for 10 minutes, and the plasma and buffy coat were removed. The RBC was washed 4 times by centrifugation at 480 g for 10 minutes in cold (4 C.) 0.85% sodium chloride (saline) solution. The RBC (100 ml.) was stored at −65 C. in plastic containers.

Steer 700, which was a carrier of the Colombian strain of *A. marginale*, was the source of infective material for calf 2, and steer 701, a carrier of the Texas strain (College Station, Texas)[d] of *A. marginale*, was the source of infective material for calf 3. Both calves were inoculated intravenously. Flasks containing 12% sodium citrate solution were used for collection of blood from the 2 donor steers. The RBC was separated from plasma and buffy coat by centrifugation and washed 4 times in saline solution prior to use as inoculum in calves. Blood samples (oxylated) were also collected from the donor steers, and the parasitemia was determined by examining Giemsa-stained blood smears. Blood samples were taken from the calves 3 times per week during the prepatent period and 1 time each day after the onset of the patent period. Thin blood smears were stained by the Giemsa technique, and PCV was measured. Blood (500 ml.) for antigen production was collected from each calf soon after the level of infection had reached 8% of parasitized RBC; calf 2 had a parasitemia of 15% and calf 3 had a parasitemia of 12% at the time of blood collection. Blood was collected in flasks containing 25 ml. of citrate solution. The RBC was separated, washed as described in the previous paragraph, and stored at −65 C.

Calf 38 was a normal calf from which blood (500 ml.) for antigen production was collected and processed as described in the preceding paragraph.

Process for Extraction of Soluble Antigens of A. marginale.—Frozen RBC from calves 1, 2, and 3 was thawed at ambient temperature. Lysed RBC from each of the calves (50 ml. from calf 1, 26 ml. from calf 2, and 34 ml. from calf 3) was used for antigen extraction. Barbital buffer (pH 7.3 to 7.4) was added to the lysed RBC (1 vol.:1 vol.), and the solutions were sonicated[e] at 4 C. for 60 seconds, using 5 ma. of current at 15-second intervals separated by 15-second rest intervals. The material was then centrifuged[f] at 100,000 g for 1 hour; the pellets (which were discarded) contained the particulate RBC stroma and parasites and the supernatant fluid, soluble antigen, and hemoglobin. The supernatant fluid was mixed with diethylaminoethyl (DEAE) cellulose to extract soluble *A. marginale* antigen by the method described by Amerault and Roby.[1]

Immunodiffusion Technique.—The medium for immunodiffusion was prepared by dissolving 1 Gm. of noble agar[g] per 100 ml. of borate buffer solution. The buffer contained 9 Gm. of boric acid and 2 Gm. of NaOH per liter of distilled water and was adjusted to a pH of 8.4. Thiomersal[h] (1:10,000 final concentration) was added. Portions (4 ml.) of the warmed (93 C.) medium were pipetted onto the microscope slides which had been coated with a thin layer of 0.1% borate buffered agar containing 0.05% glycerol. Patterns were cut into the agar, using a cork borer with outside diameter of 6 mm. The central well was surrounded by 4 circumferential wells whose perimeters were spaced 2 mm. from the perimeter of the central well. Soluble antigens I, II, and

[a] Diamond Laboratories, Mexico City, Mexico.
[b] Sorvall RC2-B Refrigerated Centrifuge, Ivan Sorvall, Inc., Norwalk, Conn.
[c] Sorvall Relative Centrifugal Force Chart, Ivan Sorvall, Inc., Norwalk, Conn.
[d] Department of Veterinary Microbiology, College of Veterinary Medicine, Texas A&M University, College Station, Texas.
[e] Branson Sonifier, Model S-15, Branson Instruments, Inc., Danbury, Conn.
[f] Beckman L-2 Ultracentrifuge, Spinco Division, Beckman Scientific Instruments, Palo Alto, Calif.
[g] Special Agar-Noble, Difco Laboratories, Detroit, Mich.
[h] Merthiolate, Eli Lilly Company, Inc., Indianapolis, Ind.

III, as well as normal (noninfected) RBC material (soluble antigen IV), were compared in circumferential wells surrounding a central well containing 1 of the 4 reference serums derived from serums of the experimental calves.

Immunoelectrophoretic Technique.—The micromethod introduced by Sheidegger[18] and outlined in an operating manual[1] was used with some modifications. The medium was prepared as prescribed for immunodiffusion technique. Holes were cut in the agar, using a cork borer with an outside diameter of 6 mm., and these were 2 mm. distant from troughs which were 2 mm. wide. Agar was removed and approximately 60 λ of soluble antigen solution was placed in the well. Electrophoretic separations[2] were made at ambient temperature with a current of 350 v. and approximately 10 ma. per tray for 1 hour. Troughs were filled with approximately 150 λ of a reference serum. Soluble antigens I, II, and III were tested with homologous and heterologous reference serums.

Serologic Examinations

Inoculation of Calves and Collection of Samples.—Blood was collected in sodium citrate solution (0.5 ml./10 ml. of blood) from calves 1, 2, and 3 which had been used for antigen production. Calves 4 through 7 were inoculated intravenously each with 5 ml. of blood from calf 2; and calves 12 through 15, with 5 ml. of blood from calf 3. Calves (No. 16 through 19) in the control group were each inoculated intravenously with 5 ml. of saline solution. Approximately 20 ml. of blood was collected from each inoculated calf on the day of inoculation and twice each week thereafter, and serum was prepared. Serum was stored at −20 C. Blood samples (oxylated) were also collected twice each week for examination for parasitemia and determination of PCV. Blood smears were stained by the Giemsa technique.

Preparation of Alum-Precipitated Antigen and Rabbit Inoculation.—The CF test[4] was performed on serums which had been prepared (2 times per week) from calves 4 through 19 after they were inoculated. Serums separated from blood which had been drawn from each calf within one week after the CF titer became maximal were used to prepare an aluminum potassium sulfate (alum-precipitated) antigen[10] for rabbit inoculation.[5] Serum from which protein was precipitated for rabbit inoculation was collected from control calves (No. 16 through 19) 34 days after they were inoculated with saline solution, this being the average time between the day of inoculation and the development of maximum CF titers in the exposed calves.

Testing (Screening) of Serum to Detect Antibody Against the Soluble Antigens.—Serum separated from blood which was collected 2 times per week from the exposed calves (No. 4 through 15) was tested in immunodiffusion systems with homologous antigen preparations (soluble antigen I, II, or III). The initial visible reaction of antigen with antibody and degree of opacity of subsequent reactions were recorded. Serums from the calves that seemed to have high concentrations of antibody, as evidenced by production of strong precipitin lines, were retained as reference serums. Serums from control calves (No. 16 through 19) were also tested with the soluble *A. marginale* antigens for the presence of antibody.

Serologic Response in Intact Calves.—Microscope slides were prepared as described in the paragraph *Immunodiffusion Technique.* Tris-barbital-sodium buffer[6] was used in the concentration of 1:1,800 (pH 8.8) for agar preparation. Patterns were cut in the agar, using an immunoelectrophoresis punch[7] with a well diameter of 1.5 mm. and trough widths of either 1.0 or 2.0 mm. Electrophoresis separations were made at ambient temperature, using a current of 450 v. and 9 ma. per tray of 6 slides.

Serum samples of the 12 infected calves (No. 4 through 15) during the acute phase of disease (as determined by CF test results) were separated electrophoretically and then tested against serum from rabbits inoculated with the respective alum-precipitated antigen, as well as against serum from rabbits inoculated with alum-precipitated antigen derived from normal

[1] Gelman Equipment for Immunoelectrophoresis and Immunodiffusion, Gelman Instrument Company, Ann Arbor, Mich.

[2] Deluxe Electrophoresis Chamber, Gelman Instrument Company, Ann Arbor, Mich.

[6] Gelman High Resolution Buffer, Gelman Instrument Company, Ann Arbor, Mich.

[7] Immunoelectrophoresis Punch Set, Gelman Instrument Company, Ann Arbor, Mich.

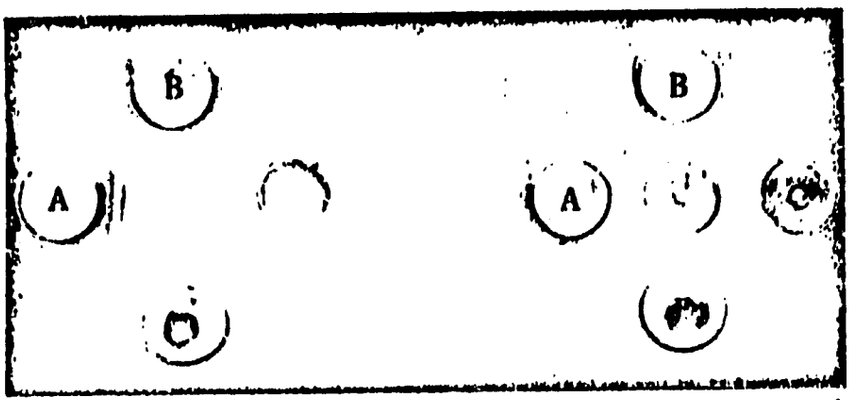

Fig. 1—Left: Centrally located reference serum from calf 4 infected with attenuated *A. marginale*. A, soluble antigen III; B, soluble antigen II; C, soluble antigen I; and D, soluble antigen IV.
Right: Reactants are same as those on left with respect to letters. Reference serum in center from calf 8 infected with the Colombian strain of *A. marginale*. After 72 hours of incubation.

bovine serum. Serums from control calves (16 through 19) were also tested against the respective hyperimmune serum from rabbits. Test serums (5 λ) were placed in the wells and hyperimmune serum (250 λ) was used in the troughs.

In another set of test patterns, serum from each infected calf and normal serum from control calves were placed in separate wells. After electrophoresis, this material was tested against serum from rabbits inoculated with the respective alum-precipitated antigen derived from serums of calves during the acute phase of infection.

Results

Soluble Antigens of A. marginale

Immunodiffusion.—Antigens I, II, and III formed one line of precipitation when tested with any of the 3 reference serums representing the various strains of *A. marginale* (Fig. 1). In each series of patterns, the formation of identity lines was evident. There was no evidence of precipitation produced by soluble antigen IV.

Immunoelectrophoresis.—All soluble

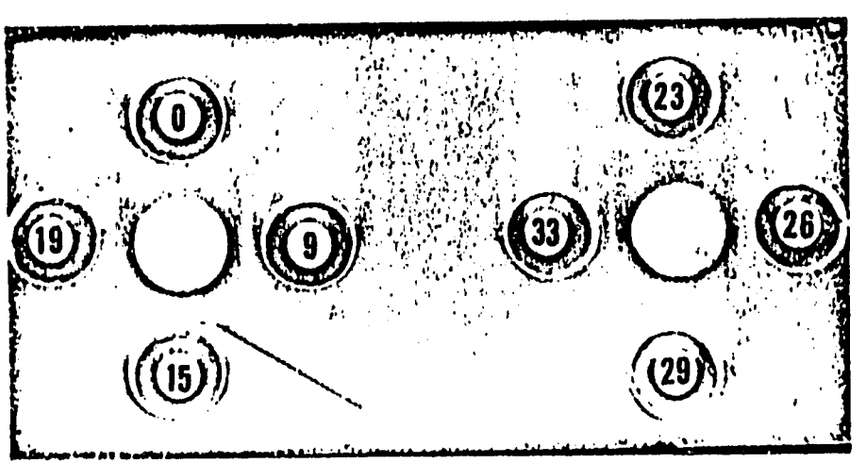

Fig. 2—Immunodiffusion reaction of centrally located soluble antigen II with serum samples from a calf inoculated with the Colombian strain of *A. marginale*. Moving clockwise from the top of patterns located at the left and continuing on the right the serum samples were obtained on the day of inoculation and at postinoculation days 9, 15, 19, 23, 26, 29, and 33. Arrow points to first visible line of precipitation. After 48 hours of incubation.

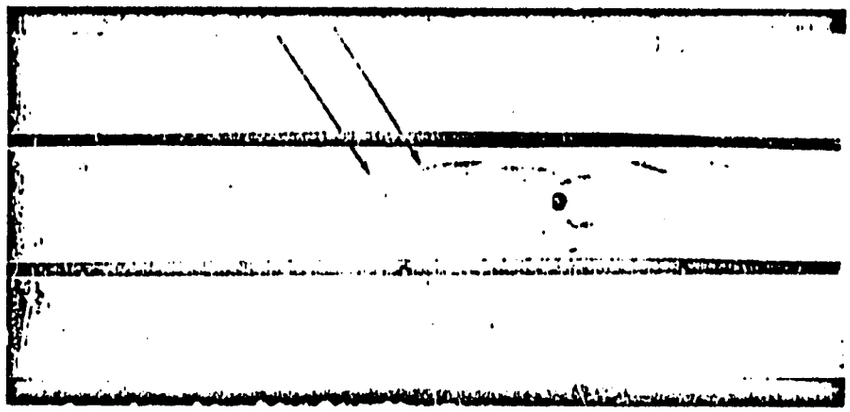

Fig. 3—Immunoelectrophoresis of serum (in well) from calf 4 during acute phase of infection with attenuated *A. marginale*. Top trough (1 mm. wide) contains hyperimmune serum from rabbit injected with alum-precipitated antigen prepared from the same serum which was exposed here to electrophoretic separation. Bottom trough contains hyperimmune serum from a rabbit injected with alum-precipitated antigen prepared from normal bovine serum. Arrows point to *gamma* globulin (left) and *beta* globulin (right) which appeared in serum from calf during acute phase, but which were shorter and indistinct, respectively, in normal bovine serum. After 48 hours of incubation.

antigens migrated approximately the same distance toward the anode and formed one arc of precipitation when tested with homologous reference serum.

Serologic Response

Serum Testing (Screening) to Detect Antibody Against the Soluble Antigens.—Antibody formed against the soluble antigen derived from the Texas strain appeared at an average of 29.8 days after inoculation (av. of 4 calves in group). Antibodies against the Colombian and the attenuated vaccinal strains appeared at an average of 25.8 days and 23.0 days, respectively, after calves 4 through 15 were inoculated with blood from carrier calves (Table 2). Concentration of antibody fluctuated as indicated by the density of precipitin lines (Fig. 2).

Serologic Response in Intact Calves.—The proteins in serums prepared from calves during the acute phase of induced disease, that were precipitated by hyperimmune serum from rabbits inoculated with alum-precipitated antigens prepared from serum of calves with acute infection or normal bovine serum, are shown (Fig. 3). Also, serum proteins of animals acutely infected with *A. marginale* and normal serum proteins are shown (Fig. 4). A *beta* globulin[3,8,9] not present in normal bovine serum was demonstrated in most serum samples prepared during acute phases of infection in calves 4 through 15. A longer *gamma* globulin arc appeared in serums from acutely affected calves, as compared to that occurring in normal bovine serum (Table 3). These protein arcs were demonstrated in serum samples from calves which were infected with each of the 3 *A. marginale strains*. Other protein components varied inconsistently in serum samples from the infected calves and normal bovine serum.

Discussion

Soluble Antigens.—The method of soluble antigen extraction used in the present experiment had been used to extract antigenic material from RBC parasitized by a strain of *A. marginale* of United States origin. There was no report, however, on the use of this method to extract soluble antigens from a Colombian strain or the attenuated organism. Reportedly,[5,6] antigenic differences may exist between the various strains of *A.*

marginale; thus the comparative experiment was undertaken. The work of Ameault and Roby[1] involved applying the extraction method to blood with 80% of RBC parasitized. The attenuated *A. marginale* strain characteristically produces a low percentage of parasitized RBC, and it was not known whether adequately potent antigen for testing by agar gel diffusion technique could be prepared from such blood. Calf 1 developed a maximal parasitemia involving 8% of RBC. Since blood was collected from calves 2 and 3 when their parasitemia was 15 and 12%, respectively, initial volumes of RBC used in the process for producing soluble antigens were reduced proportionally to equilibrate the amount of *A. marginale* protein subjected to extraction.

Soluble antigens I, II, and III were apparently identical in all characteristics that were tested. Adjacent lines of precipitation had continuity at the point where 2 lines met. Therefore, the antigens were considered identical. Further evidence of sameness was gained by immunoelectrophoretic migration of the various antigens to the same mobility zone and cross reactivity of all antigens with heterologous serum.

TABLE 3—Additional *Beta* and Lengthened *Gamma* Arcs Seen in Serum Prepared from Calves with Induced Acute Infections of *Anaplasma marginale*

Inoculum	Calf No.	Lengthened *Gamma*	Additional *Beta*
Soluble antigen I (attenuated vaccinal strain)	4	Present	Present
	5	Present	Present
	6	Present	Present
	7	Present	Absent
Soluble antigen II (Colombian strain)	8	Present	Present
	9	Present	Present
	10	Present	Absent
	11	Present	Present
Soluble antigen III (Texas strain)	12	Present	Present
	13	Present	Present
	14	Present	Present
	15	Present	Present

Serologic Response.—The serologic responses in the calf groups each infected with a given *A. marginale* strain were similar. By immunoelectrophoretic technique, 2 arcs of precipitation were usually identified in serums of calves during the acute phase of induced disease. The *beta* arc appeared only in serums of these calves and the *gamma* arc extended further toward the cathode portion.

The additional, or more extensive, protein components in serum of calves during the acute phase may be manifestations of protein present in smaller concentrations in normal bovine serums, or

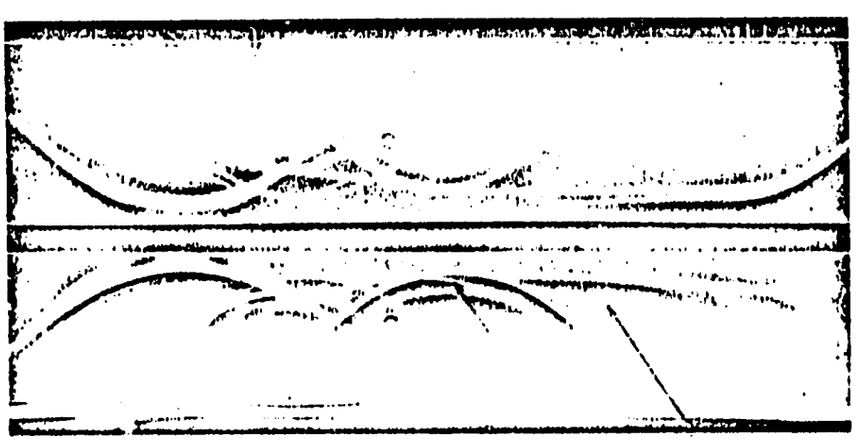

Fig. 4—Immunoelectrophoresis of serum from calf 13 during acute phase of infection with the Texas strain of *A. marginale* (top well) and serum from calf 16, a normal control (bottom well). Trough (2 mm. wide) contains hyperimmune serum from rabbit injected with alum-precipitated antigen prepared from serum used in the top well. Arrows point to *gamma* globulin (left) and *beta* globulin (right) components, which appeared in serum from calf 16 during acute phase of infection, but which were shorter or indistinct, respectively, in normal bovine serum. After 72 hours of incubation.

of normal proteins which have been altered antigenically and electrophoretically during the immune response, or of specific proteins formed during disease. The long *gamma* arc may have been caused by partial retention of the antigen by the gel or by the presence of the same antigenic group on molecules of different electrophoretic mobilities.[2] However, Murphy *et al.*[8] using immunoelectrophoresis technique, demonstrated that the *gamma* arcs are lengthened in serum collected during the course of anaplasmosis and shifted toward the trough, indicating increased concentratation. Also, lengthening may have indicated synthesis of an electrophoretically slower subpopulation of the protein.

All arcs, however, should be labeled with caution, since they can be identified with absolute certainty only by the use of absorption of specific serum components with specific immune serum or simultaneous electrophoresis of known serum components.

Results of the present experiment that pertain to the efficacy of the attenuated agent in a program of anaplasmosis control in Colombia can be evaluated only in conjunction with field studies. Recent work in this area has indicated that the attenuated agent does not protect calves against field challenge exposure in Colombia.[7]

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CONTRIBUCION AL ESTUDIO DE LA DISTRIBUCION DE GARRAPATAS EN COLOMBIA, SUR AMERICA

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INTRODUCCION

En vista de la gran potencialidad de un gran número de especies de garrapatas en la transmisión de agentes patógenos en todas partes del mundo, la necesidad de su control requiere poca o ninguna elaboración. En Colombia las Babesiosis son ocasionalmente transmitidas por las picaduras de ciertas especies de garrapatas que son y han sido por largo tiempo, responsables de pérdidas económicas en una mayor proporción de lo que anteriormente se había reconocido. Para tener éxito en las medidas de control de las garrapatas, es necesario conocer su distribución exacta y la población de especies relacionadas con la epizootiología de la Babesiosis bovina. En el momento no hay suficiente información relacionada con la población y distribución de

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garrapatas transmisoras de hematozoarios en Colombia (11). En el mundo se han hecho intentos para determinar la población de garrapatas en algunas regiones de Colombia, particularmente donde la incidencia de babesiosis y anaplasmosis son altamente prevalentes.

En condiciones naturales toda Babesia es transmisible de animales infectados a animales sanos a través de las garrapatas. Los vectores de la mayoría de las especies del mundo son conocidas y restringidas a las garrapatas de la Familia Ixodidae. La transmisión no es sólo un proceso mecánico. Los Hematozoarios pasan por un ciclo de desarrollo en el cuerpo de la garrapata, antes de que puedan ser transportados con la saliva en forma infecciosa. Este tipo de transmisión es transmisión de estado a estado o trans-estadial, cuando esto ocurre en ninfas de garrapatas o garrapatas adultas que han ingerido sangre conteniendo Babesia durante un previo estado de su desarrollo, i.e. larvas o ninfas. Algunas de las babesiosis son transmitidas "hereditariamente" o transovariamente por la garrapata hembra, la cual ha ingerido sangre de un animal infectado; también a través del huevo de la larva y también durante los últimos estados de evolución (6).

Una gran importancia se dió al estudio de las enfermedades transmitidas por las garrapatas, cuando Smith y Kilborne (10) probaron que los carrapatos Boophilus annulatus era el vector de la Babesia bigemina, la causa de la fiebre de Texas. Hay una gran variedad de agentes de las enfermedad transmitidas por garrapatas: Protozoos, Rickettsias, Virus, Espirochetas, Bacterias e inclusive un tipo intermediario de semi-

que se saben son transmitidas por miembros de la Familia Ixodidae (Murray) y Argasidae (Canestrina). Muchas especies de garrapatas de la Familia Ixodidae han sido implicadas en la transmisión de infecciones por protozoarios a los animales (3). La biología de estos protozoos dentro de artropodos huéspedes ha sido estudiada extensamente, pero muchos puntos en el desarrollo de las Babesias tanto en el huésped animal como en el vector invertebrado todavía permanecen sin dilucidar.

Los objetivos de este trabajo fueron: (1) coleccionar e identificar las especies de garrapatas que estan involucradas en la epizootiología de la Babesiosis bovina en Colombia, (2) determinar el efecto de las condiciones ambientales en la población de garrapatas y medir la incidencia de la infección de garrapatas en ciertas razas de ganado.

MATERIALES Y METODOS

Animales infectados con Babesia bigemina y Babesia argentina fueron usados como fuentes en la colección de garrapatas. El grado de infección de estos animales fué determinado por frotis delgados de sangre y pruebas de fijación del complemento. Los animales estaban localizados unos en varias estaciones experimentales del Instituto Colombiano Agropecuario (ICA): Palmira (Valle del Cauca) y Turipaná (Costa Norte); otros en fincas de propiedad particular: el Valle del Cauca, Costa Norte, Río Sumapaz y Río Magdalena. Las garrapatas fueron coleccionadas durante un año de diferentes razas: Holstein-Friesian, Cebú,

Blanco Orejinegro, Costeño con Cuernos. Los animales eran de diferentes edades comprendidas entre los 2 meses hasta los 2 años. Las garrapatas fueron recogidas de animales premunizados contra babesiosis y expuestos a la prueba de la picadura de garrapatas. Estas fueron colectadas de las diferentes partes del cuerpo de los animales y preservadas en etanol al 95%. Adultos, ninfas y larvas de ambos sexos fueron recogidas para propósitos de identificación.

RESULTADOS

Los resultado de este trabajo se demuestran en la Tabla 1.- En las granjas de ICA y fincas de propiedad privada del Valle del Cauca, las siguientes especies fueron identificadas durante 1969: Dermacentor nitens y Boophilus microplus. En fincas privadas localizadas en Fusagasugá, Río Sumapaz, Magdalena y Costa Norte, las siguientes especies fueron identificadas: Amblyomma cajennense, Dermacentor nitens y Boophilus microplus. En Enero de 1969 se colectaron garrapatas de 15 animales en la granja del ICA en Palmira. Estos animales estaban infectados con Babesia bigemina y Babesia argentina . Durante Marzo de 1969, las garrapatas fueron recogidas de los animales: Boophilus microplus y Dermacentor nitens. Durante el segundo semestre de 1969, Dermacentor nitens fué encontrado solamente en los mismos animales. Esta garrapata, Dermacentor nitens, se encontró localizada predominantemente en las orejas. En las fincas privadas del Río Magdalena Boophilus microplus se encontró en el ganado Cebú. Todos estos animales localizados en el ICA y en las fincas pri-

Tabla 1.-Colección e identificación de las distintas especies de garrapatas de animales infectados con *Babesia* spp., en Colombia.

Número de animales y localización	Fecha de colección	*Dermacentor nitens*			*Amblyomma cajennense*			*Boophilus microplus*		
		H*	M*	N*	H*	M*	N*	H*	M*	N*
Palmira (15) (Valle del Cauca)	1. 16. 69							31	7	10
Palmira (15) (Valle del Cauca)	3. 10. 69	28	15	29				10	12	63
Manizales (10) (Cauca Norte)	5. 15. 69	2	1	9				50	7	6
Palmira (20) (Valle del Cauca)	5. 29. 69	325	274	208				2	5	1
Lonzaguá (15) (Río Sumapaz)	5. 30. 69				2			6	2	3
Cali (15) (Valle del Cauca)	6. 3. 69							35	6	17
Palmira (20) (Valle del Cauca)	8. 26. 69	57	69	19						
Palmira (20) (Valle del Cauca)	9. 24. 69	117	152	121						
Lugo (20) (Valle del Cauca)	9. 28. 69	49	18	72				45	14	4
Palmira (15) (Valle del Cauca)	10. 10. 69	115	99	127						
La Dorada (10) (Río Magdalena)	11. 14. 69							12	7	5
Fusagasugá (10) (Río Sumapaz)	11. 20. 69	21	18	15						
Bucaramanga (20) (Valle del Cauca)	11. 18. 69	29	31	62				8	5	12
Girardot (30) (Río Magdalena)	1. 14. 70							392	24	35
Magdalena (20) (Costa Norte)	1. 25. 70				4	3	1	74	23	21

*H= Hembra
M= Macho
N= Ninfa

vadas fueron bañados con garrapaticidas.

DISCUSION Y CONCLUSIONES

Bajo este aspecto se pueden hacer observaciones muy interesantes; el Boophilus microplus se presentó en áreas enzooticas de Babesiosis y Anaplasmosis. Esto confirma los informes publicados previamente en cuanto a que este vector estaba involucrado en la epizootiología de la infección de las especies de Babesia en Colombia (4, 5, 8, 12). Sin embargo, el hecho de que el Dermacentor nitens y Amblyomma colombianum se encontraron en animales que se sabían infectados con Babesiosis y Anaplasmosis es un hallazgo muy interesante y el significado no es evidente. Es bien sabido que el Dermacentor nitens tiene como huésped el caballo y ha sido reportado como un vector de la Babesiosis equina en los Estados Unidos (9). La pregunta es si el bovino es un huésped accidental del Dermacentor nitens y si ñó cuál es el papel de esta garrapata en la transmisión de la Babesiosis bovina? De acuerdo con la Tabla 1 algunos ejemplares estaban parasitados solamente por Dermacentor nitens. En base a esto nos preguntamos si habrá competencia entre infestaciones por Dermacentor nitens y Boophilus microplus . Según Riek (6) las siguientes especies de garrapatas están involucradas en la transmisión de la Babesiosis bovina por Babesia bigemina (10) en el mundo: Boophilus annulatus (Say.), Boophilus calcaratus (Bir)., Boophilus decoloratus (Koch), Boophilus microplus (Can), Riphicephalus bursa (Can. y Fan.), R. appendiculatus (Neum), R. evertsi (Neum.). Las garrapatas transmisoras de Babesia major

(Sergent et.al., 1926) no son conocidas. Probablemente el Ixodes ricinus y el Haemaphysalis cinnabarina punctata están involucradas Para la transmisión de la Babesia bovis ((Babes, 1888) Syn: Babesia argentina (Lingniers, 1903), Babesia berbera (Sergent et. al., 1924) las siguientes especies están involucradas: Ixodes ricinus (Linn.), I. persulcatus (Sch.), R. bursa, Boophilus calcaratus, Boophilus microplus. Para la transmisión de Babesia divergens(M'Fadyean y Stockman, 1911) las siguientes especies de garrapatas están involucradas: Ixodes ricinus, Haemaphysalis cinnabarina punctata (Can. y Fan.)

En Colombia, Reyes (7) informó por primera vez la presencia de Dermacentor nitens en bovinos en Palmira. Mas tarde Hubar Luna(2) encontró el Dermacentor nitens en 11 haciendas de 7 municipios del Valle del Cauca, pero ninguno de ellos dió explicación al significado de estos hallazgos . En la actualidad nuestro hallazgo del Dermacentor nitens y Amblyomma cajennense en las especies bovinas en otras partes de Colombia (Montería, Río Sumapaz y Costa Norte) en grandes cantidades , muestra la urgente necesidad de hacer mas investigaciones para determinar cual es el papel de estas garrapatas en la epizootiología de la Babesiosis bovina en Colombia.

El estudio para determinar la distribución y población de las especies de garrapatas en otras partes de Colombia y para dar respuesta a otros factores ecológicos en relación con las garrapatas y su huésped sigue en proceso. Se necesita una mayor investigación antes de poder llevar a cabo un programa efectivo de control.

RESUMEN

Este estudio fué realizado como una parte del proyecto sobre Hematerozoarios durante 1969 en el Laboratorio de Investigaciones Médicas Veterinarias en Bogotá. El propósito de estos experimentos fué colectar e identificar las diferentes especies de garrapatas que están involucradas en la epizootiología de la Babesiosis bovina en Colombia. Aunque la Babesiosis bovina fué reportada en Colombia (1) no se han realizado muchos trabajos acerca de las especies de garrapatas involucradas en la transmisión de la enfermedad. Aunque el Boophilus microplus es la garrapata predominante en los climas medios y cálidos no se sabe exactamente la distribución de las garrapatas en las diferentes regiones de Colombia. Para desarrollar un efectivo programa de control, la distribución de las especies de garrapatas debe ser determinada. Este es el primer intento para lograr este objetivo.

Animales infectados con Babesia bigemina y Babesia argentina fueron usados como fuente de colección de garrapatas. La infección de estos animales fué determinada por frotis de sangre y por medio de la fijación del complemento. Los animales estaban localizados en varias granjas del Instituto Colombiano Agropecuario (ICA): Palmira (Valle del Cauca) y Turipaná (Costa Norte); también en animales localizados en fincas de propiedad privada en el Valle del Cauca, Costa Norte, Río Magdalena y Río Sumapaz. Las garrapatas fueron coleccionadas a lo largo de un año en diferentes razas: Holstien-Friesian, Cebú, Blanco Orejinegro, Costeño con Cuernos y

animales de diferentes edades. Además las garrapatas fueron colectadas de diferentes partes del cuerpo de los animales y preservadas en etanol 95%. Adultos, ninfas y larvas de ambos sexos fueron recogidas para propósitos de identificación.

Dermacentor nitens: fué encontrado en los animales infectados con Babesiosis en el Valle del Cauca, Costa Norte, Río Sumapaz; Amblyomma cajennense: se encontró en los animales infectados con Babesiosis y Anaplasmosis en el Río Magdalena y la Costa Norte. Boophilus microplus: fué encontrado en los mismos animales infectados con Babesiosis y Anaplasmosis usados en estos experimentos y en aquellos lugares donde dicho experimento fué realizado. Hasta el presente el significado de estos hallazgos no es claro y por lo tanto deberá adelantarse mas investigación al respecto.

Los experimentos siguen en progreso para determinar la distribución y la población de las especies de garrapatas en otras partes de Colombia, con el fin de investigar el papel exacto del Dermacentor nitens y Amblyomma cajennense en la epizootiología de la Babesiosis bovina en Colombia.

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IMMUNE RESPONSE OF CATTLE VACCINATED AGAINST BABESIOSIS IN COLOMBIA, SOUTH AMERICA

by

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SUMMARY

Attempts to produce co-infectious and sterile immunity in cattle against Babesia infections have been carried out by vaccinating animals with live or killed-Babesia vaccines at Palmira, Valle del Cauca, Colombia (altitude 1000 meters). Immune responses of the vaccinated animals were evaluated by several immuno-serologic methods. The degree of resistance to tick-borne challenge (Boophilus microplus naturally infected with Babesia spp.) was determined by the percentage of recovery to normal parameters used in this study.

According to the experimental design used, a total of 110 animals was divided in 5 experimental groups to ascertain the immunologic responses. The first group consisted of 20 male, 85 kg., Holstein, 3 month-old calves which were premunized with Babesia bigemina, Babesia argentina, and 4 weeks later were exposed to tick-borne (Boophilus microplus) challenge. The second group consisted of 20 male, 95 kg., Holstein, 4 month-old calves subdivided into 4 groups and vaccinated with a killed-Babesia vaccine derived from the erythrocytes and plasma, respectively, of animals acutely

infected with Babesia bigemina and Babesia argentina. The animals were inoculated with vaccine with or without Bacto-Adjuvant Complete H 37 Ra. The third group of 40 male, 80 Kg., Holstein, 3 month-old calves was divided into two sub-groups. The first sub-group consisted of 20 animals which were premunized with Babesia bigemina and Babesia argentina and 8 days later were treated with a new experimental babesiacidal drug. The second sub-group which consisted of 20 animals was simultaneously premunized with Babesia spp. and Anaplasma marginale and later treated with their respective drugs. The fourth group consisted of 20 female, 75 kg., Holstein, 3 month-old calves prophylactically treated with drug No. 4A65 and 3 weeks later exposed to Boophilus microplus naturally infected with Babesia bigemina and Babesia argentina. The fifth group consisted of 10 animals used as controls. Responses to vaccination and tick-borne challenge were evaluated by packed cell volumes, percentage of parasitemia, body temperatures, body weight, complement fixing antibody titers, general physical conditions and percent recoveries after tick-borne challenge. Results in general indicate that resistance to babesiosis can be produced by co-infectious or sterile immunity. Experiments in prophylaxis, based on residual action of the babesiacidal drug, have given consistent and satisfactory results. In the future, it may be possible to develop control programs against bovine babesiosis based on these observations. The present status of these studies will be described.

INTRODUCTION

Bovine babesiosis (Babesia bigemina)was first reported in Colombia by Carrasquilla (1899) and was later recognized as a serious tick-borne disease by Lleras (1908). In 1931, Velásquez identified Babesia argentina and Babesia major for the first time in Colombia. Bovine babesiosis was reported to have a high mortality and widespread incidence in Colombia which depended on the presence and distribution of tick vectors at various altitudes. The mortality was specially high among the imported cattle (Guerrero and Patiño 1933, Virviescas 1934, Velásquez 1938, Román 1945 and Guerrero 1956). At the present, however the exact prevalence is difficult to estimate (Todorovic, Adams and Roberts, 1969).

This paper describes the research carried out at Laboratorio de Investigaciones Médicas Veterinarias directed mainly toward the control of bovine babesiosis in Colombia with special emphasis on the problems encountered in this type of research.

MATERIALS AND METHODS

A total of 110 Holstein-Friesian, male calves with an average age of 150 + 20 days were used in this study. The experiments were carried out a the Palmira-ICA experimental farm in Valle del Cauca (altitude 1,000 meters). Randomly selected calves, were divided into five groups, according to the experimental design used to evaluate the immunoserological responses. The first group consisted of 20 male, 85 kg., Holstein-Friesian, 3 month-old calves which were premunized with Babesia bigemina and Babesia argentina and 10 weeks later were exposed to tick-borne challenge, (Boophilus microplus naturally infected with B. bigemina and B. argentina). The second group consisted of 20 male, 95 kg., Holstein-Friesian calves, 4month-old which were subdivided into four groups A, B, C, D. Four intact calves in each group were vaccinated with respective vaccines and one splenectomized calf was not vaccinated and used as control. Calves from group A and B were vaccinated with vaccine AG-S with and without Adjuvant Complete-Freund (Todorovic et al, 1968) and calves from group C and D were vaccinated with vaccine AG-E with and without Adjuvant Complete-Freund (Mahoney, 1967), respectively. The third group of 40 male, 80 kg., Holstein, 3 month-old calves was divided into two sub-group. The first sub-group consisted of 20 animals which were premunized with Babesia bigemina and Babesia argentina and 8 days later were treated with a new experimental babesiacidal drug. The second sub-group which consisted of 20 animals was simultaneously premunized with Babesia spp. and Anaplasma marginale and later treated with their respective drugs. The fourth group consisted of 20 female, 75 kg., Holstein, 3 month-old calves prophylactically treated with drug No. 4A65 and 3 weeks later exposed to Boophilus microplus naturally infected with Babesia bigemina and Babesia argentina. The fifth group consisted of 10 animals used as controls. Furthermore, several experiments were conducted to evaluate the prophylactic and therapeutic effects, dosage, route of injection, toxicity and responses of the animals injected with a new Burroughs-Wellcome* babesiacidal drug No. 4A65.

The percentage of parasitemia (P), packed cell volume (PCV), complement fixing antibody titer (AT), body temperature (T) and percentage of mortality (M) were used as the basis for comparing the reaction produced after vaccination and tick-borne challenge. In addition, experiments were carried out to identify the existing Babesia species occurring in Colombia by morphologic and immunoserologic techniques. Attempts were made to develop a sensitive and practical serologic test for detecting Babesia serum antibodies.

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* Burroughs-Wellcome Co., Tuckahoe, N. Y.

Furthemore, experiments were carried out to evaluate complement fixation techniques described by Mahoney (1962) and tube latex agglutination test (Todorovic et al, 1968) for diagnosis of bovine babesiosis.

Some attempts were made to survey the tick population in certain parts of Colombia, particularly where the prevalence of babesiosis is high. Animals infected with Babesia bigemina and Babesia argentina were used as a source of the tick collections from Valle del Cauca, Rio Magdalena and Costa Norte. Ticks were collected from different parts of the animals body and preserved in absolute alcohol. Microscopic examination of the peripheral blood smears from animals infected with Babesia was made to establish infection. The collection of ticks was made throughout the year. Adults, nymphs, and larvae of both sexes were collected for identification purposes.

RESULTS AND DISCUSSION

Animals premunized with blood collected from patent carriers of Babesia bigemina and Babesia argentina developed slight post-inoculation reactions which were manifested by parasitemia and anemia (Fig. 1). After 3 weeks, these animals recovered from the anemia, and parasitemia was not detectable. When the animals were exposed to tick-borne challenge, Boophilus microplus naturally infected with Babesia bigemina and Babesia argentina, they were highly resistant to babesiosis but all of these were infected with Anaplasma marginale. Ticks collected from these animals were identified as Boophilus microplus and Dermacentor nitens.

A control group of non-premunized intact animals was exposed to tick-borne challenge at the same time as the premunized group. These control animals became infected with babesiosis and anaplasmosis after 4 to 10 weeks exposure and 25% of them died (Fig.2). Further losses were prevented by treatment against anaplasmosis and babesiosis. The conclusion can be made that the ticks present carriers of both Babesia spp. and Anaplasma marginale and that effective control of tick-borne disease at this location cannot be made unilaterally.

A second control group of splenectomized animals, used to test the infectivity of the blood used in the premunization experiment, developed acute babesiosis from 2 to 10 days after blood-borne challenge. As a result, 90% of these animals died (Fig.3). Further losses were prevented by 2 additional treatments againsts babesiosis. The data of this experiment show that the blood used for premunization was highly infective. Additional experimentation is needed to determine the minimum amount of blood sufficient to produce infection without severe post-premunization reactions. Experiments designed to answer these questions are now in progress.

Results of the experiments to ascertain the mechanism of sterile immunity, demonstrate the possibility of producing this type of immunity by killed-Babesia vaccine, (Fig. 4 and 5). Because some animals in this experiment died from Anaplasma and helminthic parasites, results are inconclusive and specific conclusions cannot be made at this time. Part of this experiment is currently being repeated. However, results of the experiment with AG-S vaccine plus adjuvant demonstrated a high degree of protection against tick-borne challenge. No losses occurred in the vaccinated animals, however, non-vaccinated control animals died.

On the basis of the observations made with these experiments, conclusions can be drawn that Babesia antigens produce some degree of sterile immunity. To understan the exact mechanism of this type of immunity, more works needs to be done. Degree of resistance and duration of the immunity in relationship to different environmental conditions, strains, pathogenicity of Babesia organisms and quality of tick-borne challenge needs to be determined. Susceptibility and resistance of the host also plays an important role in the tropical areas and needs further study.

At the present, some of the experiments in this report are still in progress in regard to the evaluation of the new experimental drug No. 4A65. Some preliminary results are recorded in Figures 6 and 7. However, on the basis of the data of the experiments terminated, some conclusions can be drawn. The new experimental drug No. 4A65 has prophylactic activity but has also been shown to be toxic. Animals injected with 4A65 up to 46 days before challenge are highly resistant to blood-borne challenge with Babesia spp. Non-injected control animals died when exposed to this same challenge. It was found that toxic symptoms were most pronounced when 4A65 was injected intravenously at a dosage of 3 mg/kg. Two animals of 5 injected died after injection with 3 mg/kg with the following signs of reaction: excessive salivation, lacrimination, difficulty in breathing, urination and defecation. These signs were observed one minute following injection and fatal results occurred within 2 to 10 minutes.

When dosage was reduced to 1 to 2 mg/kg and the route of injection changed from I.V. to I.M., the reaction to 4A65 were much less severe. Since all of the experiments are not completed, conclusions cannot be made as to the specific dosage to be used and to the duration of protection. The experiments now in progress should answer these questions when they are terminated. Animals injected with drug No. 4A65 prophylactically,

show resistance to babesiosis, but not to anaplasmosis. It is concluded that drug No. 4A65 is highly effective against bovine babesiosis.

In January, 1969, ticks were collected from 10 animals at the ICA farm in Palmira. These animals were known to be infected with Babesia bigemina and Babesia argentina. The species of ticks (7 males and 31 females) were identified at the Parasitology Laboratory at the National University in Bogotá as Boophilus microplus. During February and March, 1969, the following ticks were collected from the same animals: Boophilus microplus: 10 females, 10 males and 57 nymphs; Dermancentor nitens: 21 females, 15 males and 29 nymphs. In May, 1969, on 2 private farms in the Magdalena River Valley, 4 female Amblyomma cajennense and 3 female and 2 nymphs of Boophilus microplus were found.

On ICA and private farms in Valle del Cauca, the following species of ticks were identified during September, 1969: 35 females, 6 males and 17 nymphs of Boophilus microplus; 49 females, 72 males and 18 nymphs of Dermacentor nitens. On October, 1969, on the ICA experimental farm in Palmira there were 109 females, 146 males and 161 nymphs of Dermacentor nitens. On the Magdalena River Valley 3 female of Boophilus microplus were found in November, 1969 and in the Fusagasugá area in the same month 2 female identified as Dermacentor nitens were found. Also in November, 1969, at Bugalagrande, Valle del Cauca, 19 males, 18 females and 62 nymphs of Dermacentor nitens, and 2 females of of Boophilus microplus were identified from Hacienda San Marcos. During the month of December, collections were again made near Girardot and Hacienda Lucerna and 75 males, 380 females and 155 nymphs of Boophilus microplus were identified.

From this survey, very interesting observations can be made. Boophilus microplus were present in the enzootic areas of babesiosis and anaplasmosis. This confirms previously published reports that this vector was involved in the epizootiology of infection of Babesia spp. in Colombia. However, the fact that Dermacentor nitens were found on animals known to be infected with babesiosis and anaplasmosis is a very interesting finding and the significance of this is not evident. It is well known that Dermacentor nitens has as its hos the horse, and it has been reported as a vector of equine babesiosis in the United States. The question remains is the bovine an accident host of Dermacentor nitens and, if not what is the role of this tick in the transmission of bovine babesiosis? These questions must be answered by additional experimental work to clarify this relationship before a control program of tick vectors can be designed.

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CURSOS SOBRE EL TEMA DE HEMATOZOARIOS

1) Identificacion de Vectores de Babesiosis Bovina
   Dr. R. A. Todorovic

2) Identificacion de especies de Babesia y desarrollo de tecnica para pruebas Serologicas en Diagnostico de Babesiosis bovina
   Dr. R. A. Todorovic

3) Ultra estructura de Babesia bigemina
   Dr. E. F. Gonzales

4) Patogenesis de Babesiosis bovina
   Dr. L. G. Adams

5) Co-infeccion e Inmunidad Esteril en Babesiosis bovina
   Dr. R. A. Todorovic

6) Quimo-profilaxis contra Babesiosis bovina
   Dr. R. A. Todorovic

7) Malaria en las aves como ejemplo de Estudio de las enfermadades Hemotopicales en el hombre y animales
   Dr. R. A. Todorovic

8) Estudio de la Patogenesis de la Anaplasmosis bovina
   Dr. L. G. Adams

9) Estudio de la Distribucion de la Anaplasmosis en Colombia
   Dr. L. G. Adams

10) Revision General de la Tripanosomiasis Bovina
    Dr. L. G. Adams

NOTA

11) Diagnostico de Trichomoniasis bovina (Pelicula en color en Ingles 16 mm.)
    Dr. R. A. Todorovic