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May, 1971

College of Veterinary Medicine

Texas A&M University

College Station, Texas

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College of Veterinary Medicine

Texas A&M University

May, 1971

Studies on Hemotropic Diseases

Progress Report

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Studies on Hemotropic Diseases

May, 1971

SUMMARY

The major emphasis of research conducted at Texas A&M has been in the area of *Anaplasma marginale*, with secondary emphasis on other hemoprotozoal agents available for studies.

The *Anaplasma* studies have been directed in three major areas. The first three experiments reported deal with treatment studies. A fourth experiment is directed primarily at a pathogenesis study concerning environmental influences on the course of anaplasmosis in splenectomized calves. Experiments 5-14 covering quite a wide range of subject matter have been directed primarily at immunological procedures, both premunization and killed antigen preparations. These latter experiments have included studies on antigens, and antigen fractionations that might also be useful from a diagnostic point of view.

Anaplasma marginale

1. Anaplasmosis Treatment Studies, Part I

This experiment involved the use of the Burroughs Wellcome drug, 356C61, (a dithiosemicarbazone), oxytetracycline, and the Burroughs Wellcome drug, 4A65.

These drugs have been used singly and in combination at varying dose levels in an attempt to eliminate *Anaplasma marginale* in the carrier animal.

Splenectomized calves were used exclusively in these trials. The drug combinations included only 2 of the 3 drugs, and reports are not included in this section of all 3 drugs being used simultaneously. No one drug, when used alone, was effective in eliminating the infection. Oxytetracycline and 4A65 used simultaneously were also ineffective. Oxytetracycline combined with 356C61 when given in 3 injections either at 24 or 48-hour intervals was successful in eliminating *Anaplasma marginale* infections. The drug level required for the elimination of infection was 5 mg/kg 356C61 and 11 or 22 mg/kg oxytetracycline. Trials using 10 mg/kg 356C61 and 11 mg/kg oxytetracycline were also highly successful in eliminating the infection. All animals not previously treated responded favorably to this treatment with evidence being obtained that the infection was entirely eliminated.

2. Anaplasmosis Treatment Studies, Part II

In these experiments 12 non-splenectomized calves carrying infection were used. Four were treated at the level of 5 mg/kg 356C61 and 11 mg/kg oxytetracycline; 4 received only the 356C61 at the rate of 5 mg/kg body weight and 4 animals remained as controls. All animals following the treatment schedules were splenectomized 70 days after the last treatment to determine at that time if the infectious organism had been eliminated. From past experience it has been shown that such procedure will produce a recrudescence of the infection if it is present. A relapse or recrudescence of infection did occur in 2 of the 4 calves treated simultaneously with 356C61 and oxytetracycline. A relapse occurred in 3 of 4 in the group 2 and all animals in the control group showed relapse. At the time this experiment was undertaken there was

some evidence that the single treatment with 356C61 may be sufficient to eliminate carrier infections, hence, this group was included. This group, however, has more often than not failed to eliminate infection. These calves had previously been treated with oxytetracycline as a part of our premunization experiment which may, in part, explain the relapses occurring in groups 1 where both the oxytetracycline and dithiosemicarbazone had been given simultaneously. The results of this experiment did not, however, entirely support the previous study in which splenectomized calves were used.

3. Anaplasmosis Treatment Studies, Part III

This experiment describes the re-treatment of splenectomized calves that had relapsed following a treatment schedule which was ineffective in eliminating anaplasmosis. There were quite a large number of animals falling into this category, and as a matter of curiosity, it was decided that a schedule of treatment previously successful, consisting of the combination of 356C61 and oxytetracycline would be administered to further support the efficacy of this treatment schedule. Of 4 calves receiving a re-treatment supposedly effective in eliminating the carrier animals, 1 relapsed, or failed to respond favorably to treatment. A second group of 7 animals were treated with 356C61 and oxytetracycline thought to be capable of eliminating the infection. Four of these 7 animals relapsed following this treatment. In every instance where relapse occurred, a previous treatment containing oxytetracycline had been given. In the 3 animals which did not relapse, oxytetracycline had not been included in the first treatment. It is believed that this is a significant finding

and may well explain the failures in intact calves previously recorded. Not all animals that have received a previous oxytetracycline treatment failed to respond to a second treatment, but in most instances failure to respond was associated with pre-treatment exposure to oxytetracycline.

4. Environmental Influence on the Course of Anaplasmosis in Splenectomized Calves

In splenectomized calves the severity of anaplasmosis, when artifically induced by the inoculation of infectious material, produced a disease which did not appear to be significantly influenced by ambient temperature. One death occurred in the barn group that was exposed to extreme summer temperatures. Slightly lower packed cell volumes were observed in the barn group, but these differences were not significant. Even though the tendency for more severe reactions to occur under heat stress were observed, it was impossible to demonstrate statistically significant differences to substantiate this finding.

5. Effects of Freezing and Storage at -65°C. on *Anaplasma* Infected Blood

Storage of infected blood of bovine origin is practical for up to 486 days with little or no loss of infectivity. Such a blood bank could be used as a source of standard inoculum or for premunization purposes.

Storage of *Anaplasma* infected blood of deer origin was equally as infectious and useful up to an average of 85 days, producing infections at least as severe as produced by infected bovine blood. It is reasonable to suspect that continued studies will reveal that the storage qualities of such blood is comparable to bovine blood. A non-bovine host as a source of infectious materials, offers distinct advantages when premunizing cattle against anaplasmosis.

6. Premunition Experiments Comparing A. marginale, A. marginale (Attenuated), and A. centrale.

These trials involved the premunition of intact calves and mature cattle with the 3 organisms mentioned. The Diamond vaccine (an A. marginale attenuated organism), Anaplasma centrale, and A. marginale (virulent).

In mature cattle marked differences were observed in animal response to these 3 organisms. A. marginale (virulent) produced significantly more severe response than did the 2 attenuated organisms. These differences proved to be highly significant in most instances. There was very little difference between response to the Diamond vaccine and Anaplasma centrale.

Among intact calves the difference in response was less marked. Once again, however, virulent A. marginale produced somewhat more severe response than did either of the 2 attenuated organisms. There were no significant differences between the Diamond vaccine and the A. centrale in these young animals. A striking feature that must be considered, however, is the fact that 30% (3 of 10) calves vaccinated with the Diamond vaccine failed to show evidence of response. In these animals premunization did not occur, and hence, these animals must be considered as fully susceptible, if later in life they become exposed to the virulent organism. Response to A. centrale and A. marginale (virulent) was 100%.

These results suggest that the Diamond vaccine should be recommended in mature cattle, but in very young intact calves a virulent premunization, or premunization with A. centrale might be preferable.

7. Influence of Treatment with Oxytetracycline on Anaplasma Premunition of Intact Calves

A total of 12 intact calves were exposed to virulent Anaplasma marginale. On day 14, 4 of these animals were treated with oxytetracycline at the level of 11 mg/kg body weight, given intramuscularly. A similar treatment of 4 additional calves was given on day 21. Four calves remained as non-treated controls. The object of this experiment was to determine whether or not treatment would reduce the severity of response to A. marginale in intact calves.

These results of these experiments failed to show that oxytetracycline, given in the dosages indicated and at the times indicated, produced any modifying effect on the course of the infection. In all 12 animals the course of the infection was extremely mild, and all animals appeared to recover equally as well. Even though the infections were mild and the parasitemias were low, a drop in packed cell volumes occurred to about 50-60% of normal. This low packed cell volume persisted an unusually long period of time in all groups.

8. Relative Animal Response to A. marginale Relapse and Re-Infection in Splenectomized Calves

With the development of techniques for the removal of the carrier infection, a number of animals have become available for re-use or re-infection. It appeared that this was an opportunity to examine a possible residual immunity or resistance in animals treated, cleaned up, and then re-infected at varying periods of time. In addition to this relapse, infections occurring on an average of 43 days

after the end of treatment were also compared for relative severity to the initial response. Calves that had been treated, with infection being removed, and then re-infected at an average of 167 days after the last parasitemia was observed showed a response to infection equal to that of the initial disease. The low packed cell volume and the high parasitemias occurring during the secondary response were not significantly different from those occurring in the initial disease. A secondary response that was significantly less severe as measured by packed cell volume and high parasitemias was seen among animals having a relapse infection 43 days after the last parasitemia.

9. Sheep Passage

Before obtaining the Diamond vaccine or the sheep adapted A. marginale, an effort was made to make serial passage of virulent Anaplasma marginale in splenectomized sheep. This was successful, however, parasitemias were very low and the degree of response in the infected sheep was hardly detectable. A series of 3 serial passages were successful, however, and there is little doubt that this approach or procedure could have been pursued if it had seemed desirable.

10. Physical Characteristics of the Infectious Agent for Anaplasmosis

This experiment involved the high speed centrifugation of Anaplasma preparations obtained from lysed, washed red blood cells and from infected plasma. Centrifugation of lysed cells for 40 minutes at 35,000 x G removed sufficient of the infectious particles, that transmission failed to occur on the inoculation of the supernatent into susceptible splenectomized

calves. Similar centrifugation of infected plasma failed to remove the infectious particles. The injection of plasma into splenectomized susceptible calves resulted in anaplasmosis.

11. *Anaplasma* Antigen Study, Part I, Antigen Fractionation and Titrations

A series of 23 different antigens and antigen fractions have been prepared in this experiment and tested for evidence of activity, both sensitivity and specificity, using a complement-fixation test model. The procedure, when uniformly followed, gives us an accurate comparison between different types of antigens. The concentration of antigenic material per gram of sediment in active antigens ranged from 7 units per gram to 2,993 units per gram of sediment. A concentration procedure such as we have used in these experiments might provide a method for the preparation of more potent and more specific antigens for vaccine use. Attempts at detecting antigen in bovine plasma were unsuccessful, as these antigens all proved to be anti-complementary.

12. *Anaplasma* Antigen Study, Part II, Reactive Characteristics of USDA Standard CF Antigen

This study employed techniques somewhat similar to those used in the previous antigen study, but starting with a USDA standard CF antigen. Antigen titers were more than doubled by sonication, but treatments with pH 10 buffers failed to significantly influence or increase CF titers. Centrifugation at high speeds removed essentially all antigenic activity, indicating that the antigen is particulate in nature.

13. Anaplasma Antigen Study, Part III, Small volume antigen production

All previous efforts at the production of anaplasmosis antigens have started with an effort to get a maximum parasitemia with as high as possible packed cell volume to improve and increase antigen yields. The approach used in this test was just the opposite, trying instead to develop a technique whereby antigens could be detected in the early stages of the disease, possibly even before the parasitemia showed up. It would be very useful to have an in vitro technique or a serologic technique to quantitate the antigenic material present in the blood at any given time throughout the course of the disease. A standard antigen preparation technique is described using 8 ml of packed, washed, RBCs. With this quantity as a standard, comparative titrations have then been made on blood samples with parasitemias ranging from 0 to 12% and packed cell volumes ranging from 11 to 27%.

A direct correlation between the parasitemias and the antigen titers was encountered. There were, however, differences between ascending and descending infections with indications that submicroscopic antigen units are present in the red cells in ascending infections which influence the antigen titers. Positive antigen titrations were obtained in animals with as low as 0.2% parasitemias. To date, all animals having a negative parasitemia were also negative on the antigen titration. A technique of calculating antigen end points based on a regression analysis was also used in this experiment. A curvo-linear regression was observed between the parasitemia and the antigen-parasitemia ratio in ascending infections.

14. *Anaplasma* Antigen Study, Part IV, Sheep Cells as a Source of *A. marginale* Antigen

The success in the preparation of antigens from low parasitemia blood led us to believe that similar antigens could be prepared from sheep blood. This proved to be correct and the preparation of serologically active antigens was highly successful. Only a few such antigens have to date been prepared, however, evidence that the relationship between antigenic units and parasitemia in sheep antigens is similar to that occurring in bovine antigens. In one instance, a complement-fixing antigen was detected in the plasma of an infected sheep in relatively high concentrations.

Babesia

1. *Babesia* Transmission by *Boophilus annulatus*, Neuvo Laredo

During the past year, the transmission studies being conducted at Nuevo Laredo were concluded. All efforts to infect white-tailed deer with *Babesia argentina* have been unsuccessful. These attempts have included 2 instances were *Babesia* infected ticks were placed in large numbers on deer. Splenectomized calf controls, infested with ticks of common origin, developed babesiosis in the usual time. No evidence of infection was detected in either of the 2 deer. Attempts to inoculate infected *Babesia* blood into an intact deer also failed to produce evidence of *Babesia* infection. A similar effort to infect a splenectomized deer by the injection of large volumes of bovine blood, carrying *Babesia*, also failed to produce evidence of infection. Ticks having undergone one life cycle on deer failed in each instance to retain infection for calves.

The significance of this work is associated with the occurrence of Boophilus annulatus ticks on the U.S. side of the Rio Grande as the result of deer movement from Mexico to the U.S. It would appear that such ticks do not carry Babesia infections.

2. Babesia rodhaini, Treatment #1, Therapeutic Studies with Ganaseg, 4A65, 356C61, and Oxytetracycline

These studies were designed primarily to compare the relative efficacy of these four drugs in the treatment of Babesia rodhaini infected mice. On day 4 and 6 following artificial exposure mice were treated with Ganaseg at the level of 4 mg/kg, 4A65 at the level of 4 mg/kg, oxytetracycline at the level of 12mg/kg, and 356C61 at the level of 5 mg/kg. Oxytetracycline and 356C61, for the most part, were ineffective in the treatment of Babesia infection in mice. Ganaseg and 4A65, on the other hand, were highly effective. The 4A65 showed obvious evidence of superiority over Ganaseg in the treatment of Babesia rodhaini infections.

3. Babesia rodhaini, Treatment #2, The Effect of Treatment with Ganaseg and 4A65 on the Babesia Carrier

The drug 4A65 was effective in the removal of all evidence of carrier infection. Ganaseg, on the other hand, did not remove carrier infection. The treatment referred to was the same as used in the previous experiment. When these mice were challenged, the group having received Ganaseg were for the most part resistant to challenge. Those animals having received 4A65 and which had become sterile, in so far as carrier infections were concerned, showed approximately a 50% susceptibility rate to infection. It is significant, however, that almost half of the animals having been cleared up with the drug 4A65 were resistant to challenge, hence, carrying a degree of sterile immunity.

4. B. rodhaini, Treatment #3, Drug Prophylaxix Against B. rodhaini in Mice, Part 1

In this experiment 25 mice were injected 1 time with 4A65 at the rate of 2 mg/kg, and 25 mice injected 1 time with Ganaseg at the rate of 4 mg/kg. On the same day a group of 5 mice in each treatment group were infected with Babesia rodhaini. Similar exposures were made on days 4,7,14, and 21 to determine evidence of drug prophylaxis. A degree of drug response was observed from those animals challenged on day 0. The response of those animals challenged on day 4 was minimal but was detectable. Beyond this point drug treatment had no effect on the development of babesiosis. No prophylaxis could be detected by this method.beyond 4 days.

5. Drug Prophylaxis Against B. rodhaini in Mice, Part 2

A second part of this experiment used varying drug levels with a constant challenge having been given at 7 days. The drug levels on Ganaseg were 2,4, 8,16,32, and 64 mg/kg. The levels for 4A65 were 2,4,8,16, and 32 mg/kg. A constant challenge was given on day 7 to all mice. Protection was aforded by the 64 mg/kg in the instance of Ganaseg. No detectable protection could be detected 7 days after treatment when challenge was given to mice receiving the 2,4,8,16, and 32 mg/kg of Ganaseg. Very little protection was aforded mice receiving the 2 mg/kg level of 4A65. Those receiving 4,8,16, and 32, however, did show significant protection against the challenge inoculum when compared to controls.

6. Babesia rodhaini Duration of Immunity Following Premunition

This experiment was designed to measure the duration of immunity following treatment of Babesia rodhaini infected mice with 4A65 and Ganaseg.

Mice treated with 4A65 at a time when they were infected with Babesia lost the infection. Mice treated with Ganaseg retained the infection and

remained carrier animals. There were losses among the Ganaseg treated group. A progressive challenge, starting on day 14 and continuing on days 28,42,84, 112, and 140 days were made. Carrier infections in the Ganaseg group did not persist beyond the 112th day. Carrier animals were, for the most part, immune to challenge. A fairly high level of sterile immunity was observed in the 4A65 group up to 56 days. Thereafter, this immunity dropped rapidly.

7. Babesia rodhaini, Pathogenesis #1, Relationship of PCV and Parasitemia

Using 40 infected mice that had been treated with Ganaseg and 4A65, a correlation analysis between PCV and parasitemias was attempted.

A significant regression coefficient of -2.3 was observed relating PCV to parasitemia. The correlation coefficient (r) was 0.663, which with 39 degrees of freedom, is highly significant. As the parasitemia goes up the packed cell volumes are generally lower.

8. Babesia rodhaini, Pathogenesis #2, Comparative Blood and Spleen Titers

Splenic material is a good source of infectious material. While not so high a titer as blood from acute babesiosis, it is highly infective having a titer of $10^{-7.67}$. Grinding of infective blood in the Vir-tis was responsible for almost a log drop in titer. When this factor is considered, the spleen is not much lower than blood in the number of infectious units present.

9. B. rodhaini Adjuvant Vaccine Experiment

Two antigens out of 8 tested were shown to have specific protective qualities. These antigens were heavy sediments following water lysis of

infected red cells. A degree of non-specific protection, however, was noted in all antigens as well as the veronal buffer control. This is not understood, but there is some suggestion that possibly an inteferon type response is being produced by vaccination with adjuvants.

10. Toxicity Studies in Mice, 4A65 and Ganaseg

Toxicity LD 50's were calculated using 5 mice per point and 2 fold concentrations beginning at 2 mg and extending to 256 mg/kg body weight. The LD 50's for 4A65 was 49.3 mg/kg. The LD 50 for Ganaseg was 89.2 mg/kg.

Theileri cervi

1. Antigen Titration and Fractionation

A total of 4 antigens were prepared and tested. Three of these antigens were water lysed preparations in which sediments of different weights were selected and tested. The 4th antigen was a CO_2 saturated distilled water antigen. The greatest concentrationsof antigenic material was once again found in the heavy sediment of the water lysed material. The CO_2-water lysed material, while active, showed evidence of considerable denaturation.

Trypanosoma theileri

1. Trypanosoma theileri Transmission attempts and serial transfer on artificial media (In cooperation with Dr. T.J. Galvin).

Transmission attempts using infected culture material from blood agar slants failed to produce evidence of T. theileri infections in a splenectomized calf. Successful transmission was achieved by the inoculation of splenectomized

calves with blood from known T. theileri carrier cows. These calves did not show a T. theileri parasitemia on thin blood smears, but culture of their blood was positive for the trypanosome. No evidence of drops in PCV or other pathologic changes could be detected in association with this infection. In one calf, infection was detected only at days 4 and 5. Thereafter, all cultures were negative. On the other calf, positive cultures were obtained at 6 hour, 3,4,5, and 6 days, and at 22,25,29 and 32 days; thereafter, all cultures were negative.

From these studies it is obvious that the passage and transfer of Trypanosoma theileri is not easy. The means by which animals become naturally infected are not known, but probably involves more than the mechanical transfer of infected blood.

Babesia rodhaini

1. Babesia rodhaini Infectivity and immunogenicity of irradiated Babesia rodhaini (J.P. Bishop and K.L. Kuttler)

Babesia rodhaini parasitized mouse blood was exposed to 10,000 r, 14,000 r, 18,000 r, 22,000 r, and 30,000 r. Mice infected with Babesia rodhaini parasitized blood exposed to doses up to and including 22,000 r developed progressive parasitemias which were delayed in comparison to control mice. Some mice receiving parasitized blood irradiated at 26,000 r did not develop progressive parasitemias. Progressive infections were prevented by exposure to irradiation at 30,000 r. Nine out of 28 mice having received the irradiated parasitized blood survived challenge, whereas, 20 out of 20 control mice died.

Anaplasmosis Treatment Studies Part I

Introduction

Previous work reported in the 1969 and 1970 Annual Reports showed a new product, designated 356C61*, to have merit in treating anaplasmosis. This drug, a dithiosemicarbazone, was found to be equally as effective as oxytetracycline, with some evidence to suggest that it was more effective in the dose range tested. A serious drawback to this material was its toxicity. When intravenous (I.V.) injections were given daily for 10 consecutive days at the rate of 5 mg/kg, death usually occurred in a matter of days or in some instances weeks after therapy was discontinued. Such animals showed varying degrees of rumen atony, chronic bloat, and inappetence. Death in most instances could be attributed primarily to bloat. Injections of 356C61 for 5 consecutive days at the same level did not produce fatal toxic effects.

A single inoculation of the drug appeared effective in reducing parasitemia, and/or extending the incubation time depending on when administered in relation to the development of anaplasmosis in the individual animal. The multiple injection for 10 consecutive days appeared to eliminate <u>Anaplasma marginale</u> from the carrier calf, but 5 injections over a 5 day period failed to eliminate infection.

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* Burrough Wellcome Laboratories

Taking into consideration the toxic characteristics of the drug and the apparent need for prolonged drug blood levels to eliminate infection, experiments were undertaken in which multiple injections of 356C61 were spaced for varying intervals ranging from 1 day to 2 weeks, with a total number of injections being limited to 5 or less. In every instance a recrudescence of infection was detected following treatment.

During the past year efforts to eliminate the infection by combined therapy including 356C61, oxytetracycline and 4A65 (Burrough-Wellcome Co., 3,3 - Bis -(2-imidazolin-2-yl) - carbanilide dihydrochloride) have been made. These studies are continuing, and this report should be considered confidential, preliminary in nature, and incomplete as of this time.

All experimental calves used in these trials were bled at least 2 times a week and more often, if indicated, with routine observation of PCV, CF, and stained blood smears for evidence of parasitemia.

Group I: A group of 9 splenectomized calves carrying *Anaplasma* infections ranging from acute to chronic latent infections were treated with 356C61 and oxytetracycline. These were Holstein type dairy calves averaging 9 months of age in which anaplasmosis had been artifically induced by various means. Their infected status was determined by the presence of *A. marginale* in the red blood cell as seen on Giemsa stained blood smears and by a positive complement-fixation (CF) test reaction to the standard USDA *A. marginale* antigen. In most instances a depressed packed red cell volume (PCV) was evident.

At the time of treatment these calves had an average PCV of 17%, a parasitemia of 10.5%, and a CF titer of 1:93. Treatment consisted of 11 mg/kg oxytetracycline and 5 mg/kg 356C61 in 5 of the 9 calves. Four calves received 22 mg/kg oxytetracycline and 5 mg/kg 356C61. These two drugs were mixed in 150-200 ml sterile 0.85% NaCl (PSS) and injected I.V. Three injections were given all calves; 7 at 48 hour intervals and 2 at 24 hour intervals.

The results of this treatment are summarized in Table 1. Two weeks after treatment the average PCV was 28%, significantly greater than the pre-treatment PCV, and there was no evidence of an *A. marginale* parasitemia. All animals were observed for at least 120 days and none showed diagnostic evidence of a relapsing *Anaplasma* infection. Infectivity trials were conducted on 8 of the 9 calves by inoculating I.V. 200 ml freshly drawn, citrated blood from the calf being tested into a susceptible, non-infected

splenectomized calf. In no instance was A. marginale recovered by this means in the check calf. In every treated calf the CF titer dropped below the 1:5 level and, in most instances, returned to a negative state. The time required for the CF test to drop below the diagnostic level of 1:5 was highly variable ranging from 20 to 117 days, with an average of 65.4 ± 30 days. In some treated calves an unexplained temporary increase in CF titer was detected, which later disappeared. This evidence is highly suggestive that A. marginale sterilization has occurred in splenectomized calves treated as indicated.

No evidence of drug toxicity occurred in any treated animals.

Group II: A group of 8 splenectomized calves carrying Anaplasma infections similar to those in Group I were similarily treated with 356C61 and oxytetracycline, except that 356C61 was given in a larger dosage.

All calves were Holstein type, averaging 10 months of age in which A. marginale infections had been induced. At the time of treatment these calves had an average PCV of 17%, a parasitemia of 9.1% and a CF titer of 1:40. Treatment consisted of 11 mg/kg oxytetracycline and 10 mg/kg 356C61 in 7 of the 8 calves. One calf received 22 mg/kg oxytetracycline and 10 mg/kg 356C61. These 2 drugs were mixed in PSS in the amounts indicated and injected I.V. Three I.V. injections were given all calves, half at 48 hour intervals, and half at 24 hour intervals.

The results of this treatment are summarized in Table 1. Two weeks after treatment the average PCV was 26%, significantly greater than the pre-treatment PCV, and there was no evidence of an A. marginale parasitemia. Six of the 8 calves have failed to show any signs of relapsing infections for over 120 days. These same calves have been checked for infectivity

as previously described using 200 ml whole blood injected I.V. into a splenectomized calf. In no instance has infection been recovered in the check calves. Of the 2 remaining calves, 1 died of causes other than anaplasmosis 60 days after the treatment, and observations are being continued on the other. One of these 2 calves (267) died 61 days after the last treatment from acute typanites. This animal had not shown a diagnostic parasitemia during the post-treatment period. Ten days after treatment this calf was found down, caught in a gutter and unable to rise. Treatment for bloat was required at this time and, in addition, dextrose was given I.V. No further treatment was required, but the animal remained unthrifty, with periodic inappetance and a mild chronic bloat. Death occurred with no warning during the night, and necropsy failed to show any gross pathologic changes other than what was associated with severe ruminal typanites.

The serum CF titers were observed to drop the diagnostic 1:5 level in all animals, but once again this was highly variable ranging from 23 to 120 days with an average of 56.5 $\pm$ 35 days. This period of time was slightly less than the 65.4 days observed in Group I, but the differences are not significant.

As with Group I the evidence is highly suggestive that A. marginale sterilization has occurred following this treatment. The death of Calf 267, while not entirely conclusive, is indicative of drug toxicity. Past experience indicates that the drug effect is more rapid, but the first signs of bloat were seen within the period when toxic reactions could be expected. At the time the bloat was first noticed and treated it was thought to be associated with the trauma of being caught in the gutter

and being down on its side throughout the night, but the persistence of a chronic bloat suggests that more was involved. The other 7 calves did not show any evidence of drug toxicity.

Group III: A group of 9 splenectomized calves carrying *Anaplasma* infections similar to those in Group I were treated with 356C61 and oxytetracycline in the dose range used before, but giving only 2 injections instead of the previous 3.

All calves were Holstein type, averaging 11 months of age, in which *A. marginale* infections had been experimentally induced. At the time of treatment these calves had an average PCV of 16%, a parasitemia of 4.9%, and a CF titer of 1:47. Four calves were treated with 5 mg/kg 356C61 and 11 mg/kg oxytetracycline injected I.V. two times; 3 at a 24 hour interval and 1 at a 96 hour interval. Five calves were treated with 10 mg/kg 356C61 and 11 mg/kg oxytetracycline injected I.V. two times at a 48 hour interval. These 2 drugs were mixed in PSS before infection as described previously.

The results of this treatment are summarized in Table I. Two weeks after treatment the average PCV was 26%, significantly greater than the pre-treatment PCV. At that time there was no evidence of an *A. marginale* parasitemia. Of the 9 treated calves 8 showed an *A. marginale* relapse 29 to 61 days after the last treatment, with an average of 43 $\pm$ 9 days. The relapse was characterized by the appearance of a diagnostic *Anaplasma* parasitemia of 0.5% or greater, an increase in CF titers, and a drop in PCV. Relapse parasitemias were high in most instances, and comparisons between initial and relapse reactions will be presented in another section of this report. One calf, 419, failed to relapse, and lost a 1:5 CF titer

12 days after last treatment. An infectivity trial on this calf is not yet complete, but the evidence is indicative that A. marginale sterilization has occurred.

Group IV: A group of 2 splenectomized calves carrying Anaplasma infections similar to those in Group I were treated with oxytetracycline and 4A65. Both calves were Holstein type with an average age of 10 months. At the time of treatment these calves had an average PCV of 16%, a parasitemia of 5.1%, and a CF titer of 1:160. Each calf was treated with 11 mg/kg oxytetracycline and 2 mg/kg 4A65 three times at 24 hour intervals. The drugs were mixed in PSS and injected I.V. as previously described.

The results are summarized in Table 1. Two weeks after treatment the average PCV was 26 as compared to 16 before treatment. At this time no parasitemia was noted. Both animals showed an A. marginale relapse 17 days after the last treatment.

Even though 4A65 dissolves readily in water or PSS to give a clear solution, the addition of Liquamycin results in a dense, white, milk suspension.

Temporary discomfort including profuse salivation and dyspnea were noted in all treated animals, but complete recovery apparently occurred within 30 minutes. No other toxic symptoms were noted.

Group V: A group of 3 splenectomized calves carrying Anaplasma were treated with oxytetracycline and 356C61. In this instance each of the 3 calves received 1 injection of 5 mg/kg 356C61 on day 1, and 3 injections of 11 mg/kg oxytetracycline on day 1-3-5, (48-hour intervals). The drugs were mixed and diluted in 150 ml PSS prior to injection. At the time of treatment these calves had an average PCV of 23%, a parasitemia of 2.1%, and a CF titer of 1:50.

The results are summarized in Table 1. Two weeks after treatment the average PCV was 24, which was not significantly different than the pre-treatment level. One of the 3 calves showed a 0.3% parasitemia 2 weeks after treatment, even though the others were negative. All animals showed an Anaplasma relapse which occurred on an average of 34 days $\pm$ 7.0 after the last treatment.

Group VI: Group VI consisted of 5 Holstein type, splenectomized calves carrying Anaplasma which were treated 3 times at 48-hour intervals with 356C61 only. In 4 calves 356C61 was given at the rate of 10 mg/kg, and in 1 the rate was 5 mg/kg. The drug was diluted in 150 ml PSS before the I.V. injection. At the time of treatment these calves had an average PCV of 18%, a parasitemia of 5.4%, and a CF titer of 1:23.

The results of treatment are summarized in Table 1. Two weeks after treatment the PCV was 27% which represents a significant ($P<0.01$) increase. At this time all animals showed a negative parasitemia. Anaplasma relapse occurred in 4 of the 5 treated animals on an average of 49 $\pm$ 7 days. The fifth calf, 422, showed no signs of relapse in over 120 days, and was checked for infectivity by inoculating a susceptible, splenectomized calf with 200 ml citrated blood from 422. No signs of infection was noted in the test calf, and it was assummed that Anaplasma had been eliminated as the result of treatment in 422. Calf 422 had received 10 mg/kg.

No evidence of toxicity was observed in any of the 5 calves.

Group VII: Group VII consisted of 3 Holstein type, splenectomized calves carrying *Anaplasma*, which were treated 3 times at 48 and 24 hour intervals with oxytetracycline only. Two calves received 22 mg/kg I.V. 3 times at 48-hour intervals, and the remaining calf was treated with 11 mg/kg I.V. 3 times at 24-hour intervals. The drug was diluted in 150 ml PSS before the I.V. injection. At the time of the treatment these calves had an average PCV of 14%, a parasitemia of 1.7%, and a CF titer of 1:13.

The results of treatment are summarized in Table 1. Two weeks after treatment the average PCV was 24% which represents a significant ($P<0.05$) increase. A parasitemia was present in 1 of the 3 at this time. An *Anaplasma* relapse was observed in all animals an average of 19 ± 5.0 days after treatment.

No evidence of drug toxicity was noted in any of the calves.

Group VIII: Group VIII consisted of 4 Holstein type, splenectomized calves carrying *Anaplasma*, which were treated 3 times at 48-hour intervals with 4A65 only. Each animal was inoculated with 2 mg/kg 4A65 I.V. The drug was diluted in PSS before the inoculation. At the time of treatment these calves had an average PCV of 16%, a parasitemia of 3.4%, and a CF titer of 1:28.

The results of treatment are summarized in Table 1. Two weeks after treatment the average PCV was 22% which was a marked increase in PCV, but this difference was not sufficiently consistent to reach significance. An *Anaplasma* parasitemia was not evident in any of the 4 calves 2 weeks after treatment. An *Anaplasma* relapse was observed in all animals an average of 30 ± 7.0 days after treatment.

Temporary discomfort including profuse salivation, varying degrees of dyspnea, were noted in all animals. In some instances these changes were accompanied by the complete collapse of the animal. In no instance, however, were these changes permanent, and usually in 30 minutes the calf appeared normal.

Group IX: A group of 3 Holstein type, splenectomized calves carrying *Anaplasma* infections were treated with 356C61 only, 4 times at 72 hour intervals. At the time of treatment the average age was 6 months, PCV 16%, parasitemia 3.6%, and CF titer 1:200. The drug was administered at the rate of 5 mg/kg I.V.

The results of this treatment are recorded in Table 1. Two weeks after treatment the average PCV was 27%, which was significantly ($P<0.01$) greater than pre-treatment level. At this time there was no parasitemia. An *Anaplasma* relapse occurred in all animals an average of 40 ± 3 days.

No evidence of toxic reactions were observed.

Group X: Three Holstein type splenectomized calves carrying *Anaplasma* infections were treated with 356C61 only, 3 times at 7 day intervals. The treatment level was 5 mg/kg in all animals. At the time of treatment the average age was 7 months, the PCV 16%, parasitemia 2.7%, and a CF titer of 1:160.

The summary results of this treatment are recorded in Table 1. Two weeks after treatment the average PCV was 30%, which was significantly ($P<0.05$) higher than the pre-treatment level. At this time no *Anaplasma* parasitemia was detected. An *Anaplasma* relapse occurred in all calves an average of 34 ± 16 days after the last treatment.

No toxic side effects were observed in these calves.

Conclusions and Discussions:

The addition of oxytetracycline and 356C61 to relatively large volumes of PSS reduced the number of undesirable inoculation reactions when these drugs were administered intravenously. The actual time required for treatment was not significantly greater and, in some instances, less since the drugs can be given much faster in the diluted form. Usually a 14 gauge needle with a regular intravenous outfit was used, giving the drugs as rapidly as possible. Adverse reactions were rare. It is probable that an I.M. route for 4A65 would produce fewer or less severe side effects than were seen when this drug is given I.V.

A comparison of groups and group response to therapy as shown on Table 1 does show some significant trends and useful information relative to the treatment of anaplasmosis.

The number of animals, while not as great as desired, were sufficient with 1 exception (Group IV) to allow statistical evaluation. The age variation within groups was such that very little significance could be detected from this point of view. Group IX and X were exceptions to this in that they were significantly younger than some of the other groups. The pre-treatment values of PCV and parasitemia were not significantly different among any of the groups. The average 1:13 pre-treatment CF titer observed in Group VII was significantly lower than titers seen in Groups IV, IX, and X, otherwise CF average titers, while quite different, were not significantly so.

The most obvious and striking feature of these experiments was the apparent elimination of infection by combined 356C61 and oxytetracycline therapy when administered 3 times at either 24 or 48-hour intervals. Similar treatment, but for only 2 inoculations at a 24 or-48 hour interval, failed to eliminate infection in 8 of 9 animals. The only other treatment schedule to apparently eliminate infection was Group VI where double the usual 356C61 dosage given 3 times prevented a clinical relapse in 1 of 4 calves.

In nearly all treated groups a significant increase in PCV was noted 2 weeks after treatment. The only exceptions were Groups IV, V, and VIII. Animals of these groups received oxytetracycline + 4A65 3 times, 1 treatment 356C61 + 3 treatments with oxytetracycline, and 2 or 3 treatments with 4A65 only. All groups receiving 2 or more inoculations of 356C61 showed marked increases in PCV. Group VII receiving oxytetracycline for 3 times showed an increase in PCV, but this was not so impressive as in other groups.

The parasitemia had disappeared in most treated animals at the 2 week post treatment observation. Only 2 calves failed to clear up in this respect; one calf in Group VII, receiving oxytetracycline only, and 1 calf in Group V which had received 1 356C61 treatment and 3 oxytetracycline treatments.

The average relapse time is also suggestive of the relative efficacy of the various drugs. Animals receiving 356C61 generally showed longer relapse periods. In Groups IV and VII where oxytetracycline + 4A65 and oxytetracycline only were given, the relapse times were much shorter, and in the case of Group IV were significantly so. The longer relapse periods were associated with 356C61 therapy.

Of the 3 drugs used, 356C61 appears to be the most effective, even though favorable response occurred with both 4A65 and oxytetracycline. The need for a combination of 356C61 and oxytetracycline suggests a synergistic effect since even double doses of 356C61 or oxytetracycline were most often unsuccessful in eliminating infection.

A hazard of drug toxicity with 356C61 does occur when 3 or more injections at the 10 mg/kg level are given. This has occurred only 1 time, but considering the numbers used represents an unacceptibly high percentage loss. The 5 mg/kg level does appear effective, and so far has not produced signs of toxicity. Trials are now underway using lower levels of 356C61 in conjunction with oxytetracycline and/or 4A65.

Table 1

Summary of Treatment Results Using Anaplasma Infected Splenectomized Calves

	Animals		Pre-Treatment Data			Post Treatment Data					
	No.	Age (mo.)	PCV	Paras.	CF	PCV	Sig. of PCV Change	No. showing Relapsing Parasitemia	Avg. Relapse Time	SD for Relapse Time	Days Required for < 1:5 CF to occur
Group I	9	9	17	10.5	1:93	28	P<0.01	0	NT	NT	65.4 ± 30
II	8	10	17	9.1	1:40	26	P<0.01	0	NT	NT	56.5 ± 35
III	9	11	16	4.9	1:47	26	P<0.01	8	43 da.	± 9.0	12.0
IV	2	10	16	5.1	1:160	26	NS	2	17 da.	± 0	NT
V	3	9	23	2.1	1:50	24	NS	3	34 da.	± 7.0	NT
VI	5	14	18	5.4	1:23	27	P<0.01	4	49 da.	± 7.0	45.0
VII	3	10	14	1.7	1:13	24	P<0.05	3	19 da.	± 5.0	NT
VIII	4	12	16	3.4	1:28	22	NS	4	30 da.	± 7.0	NT
IX	3	6	16	3.6	1:200	27	P<0.01	3	40 da.	± 3.0	NT
X	3	7	16	2.7	1:160	30	P<0.05	3	34 da.	± 16.0	NT
Significance		P<0.01	NS	NS	P<0.05*	P<0.05			P<0.01		
DRS		5.1				6.0			21		

NT: No test - or not applicable.
DRS: Difference Required for Significance.
*: The CF Titer 1:13 in Group VII was significantly lower than titers occurring in Groups IV, IX and X. No other significant differences in CF titer was detected.

Table I (continued)

Group I: 5 mg/kg, 356C61 and 11 or 22 mg/kg oxytetracycline, I.V. 3 times at 24 or 48 hour intervals.

II: 10 mg/kg, 356C61 and 11 or 22 mg/kg oxytetracycline I.V. 3 times at 24 or 48 hour intervals.

III: 5 or 10 mg/kg 356C61 and 11 mg/kg oxytetracycline I.V., 2 times at 24-48 or 96 hour intervals.

IV: 11 mg/kg oxytetracycline and 2 mg/kg 4A65 I.V., 3 times at 24 hour intervals.

V: 5 mg/kg 356C61, I.V. - 1 time, and 11 mg/kg oxytetracycline I.V., 3 times at 48 hour intervals.

VI: 5 or 10 mg/kg 356C61 I.V., 3 times at 48 hour intervals.

VII: 11 or 22 mg/kg oxytetracycline I.V., 3 times at 48 hour intervals.

VIII: 2 mg/kg 4A65 I.V., 2 or 3 times at 48 hour intervals.

IX: 5 mg/kg 356C61 I.V., 4 times at 72 hour intervals.

X: 5 mg/kg 356C61 I.V., 3 times at 7 day intervals.

Figure 1

Group 1

9 Splenectomized Calves Treated 3 Times at 24 or 48 hour Intervals with: oxytetracycline - 11 mg/kg
356C61 - 5 mg/kg

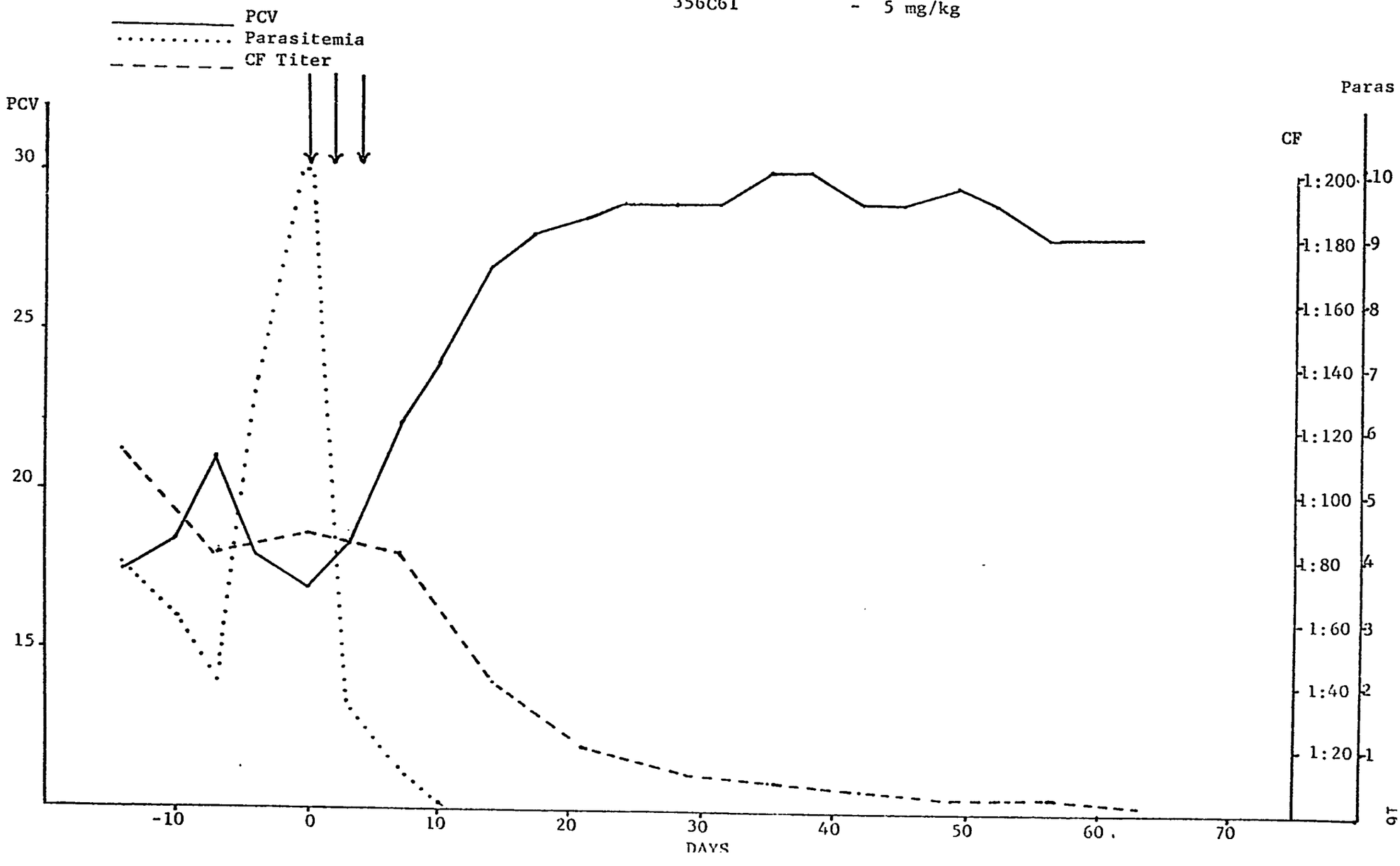


Figure 2

Group 2

8 Splenectomized Calves Treated 3 Times at 24 or 48 Hour Intervals with:
Oxytetracycline - 11 mg/kg
356C61 - 10 mg/kg

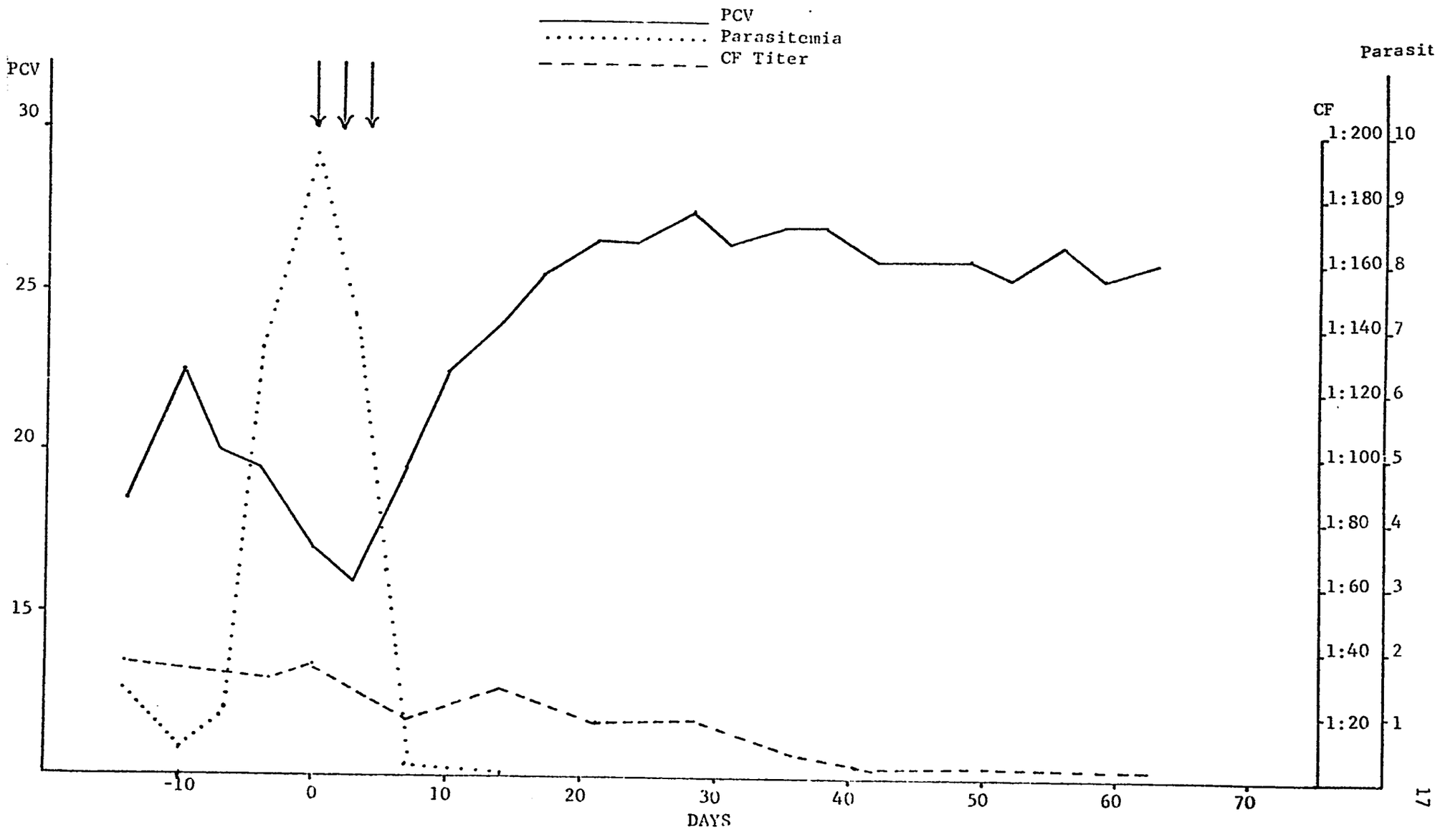


Figure 3

Group III

9 Splenectomized Calves Treated 2 Times at 24 or 48 Hour Intervals with:
oxytetracycline - 11 mg/kg
356C61 - 5 or 10 mg/kg

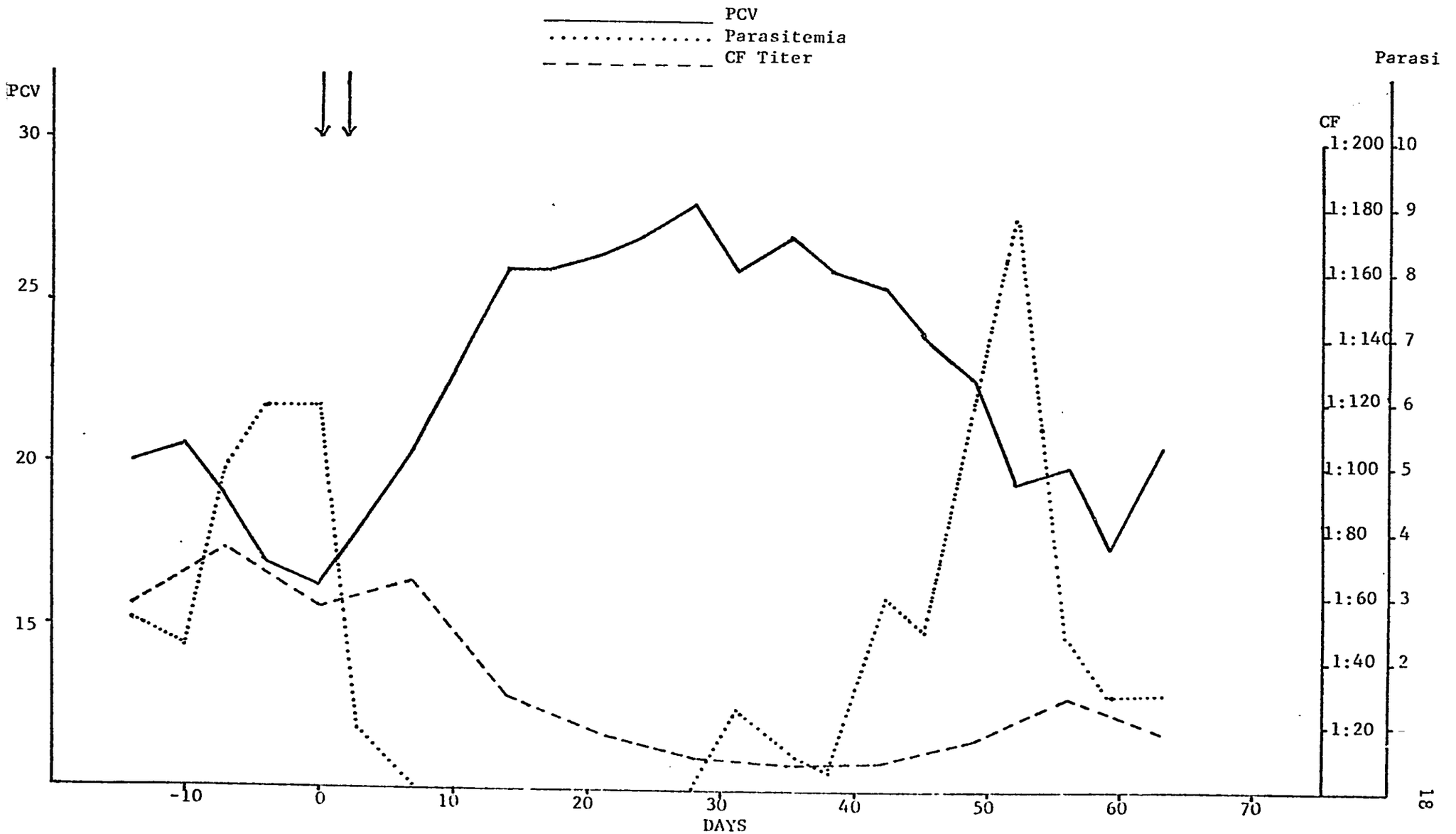


Figure 4

Group IV

2 Splenectomized Calves Treated 3 Times at 24 Hour Intervals with:
oxytetracycline - 11 mg/kg
4A65 - 2 mg/kg

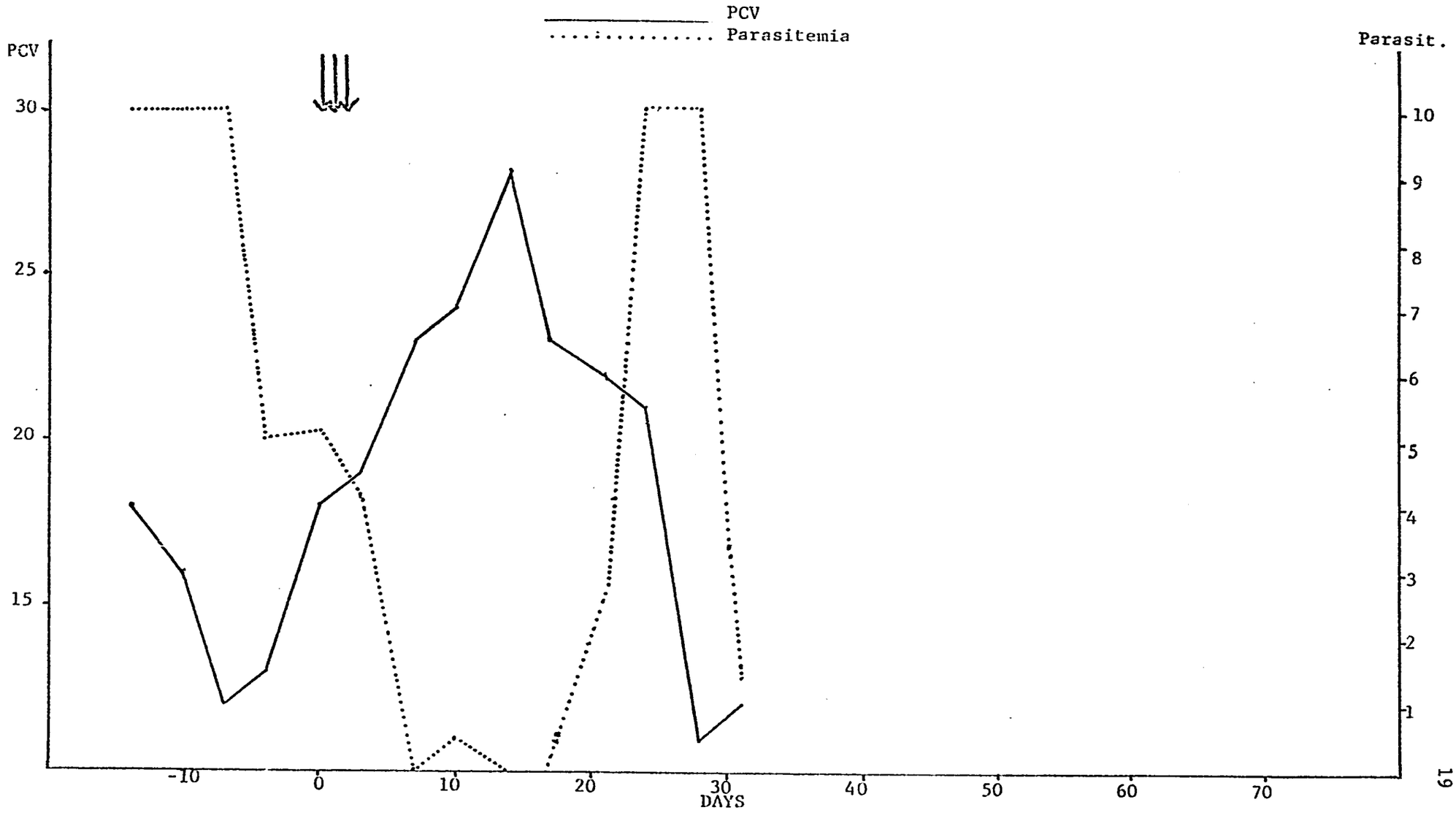


Figure 5

Group V

3 Splenectomized Calves Treated 1 Time with 5 mg kg 356C61
and 3 Times with 11 mg/kg Oxytetracycline

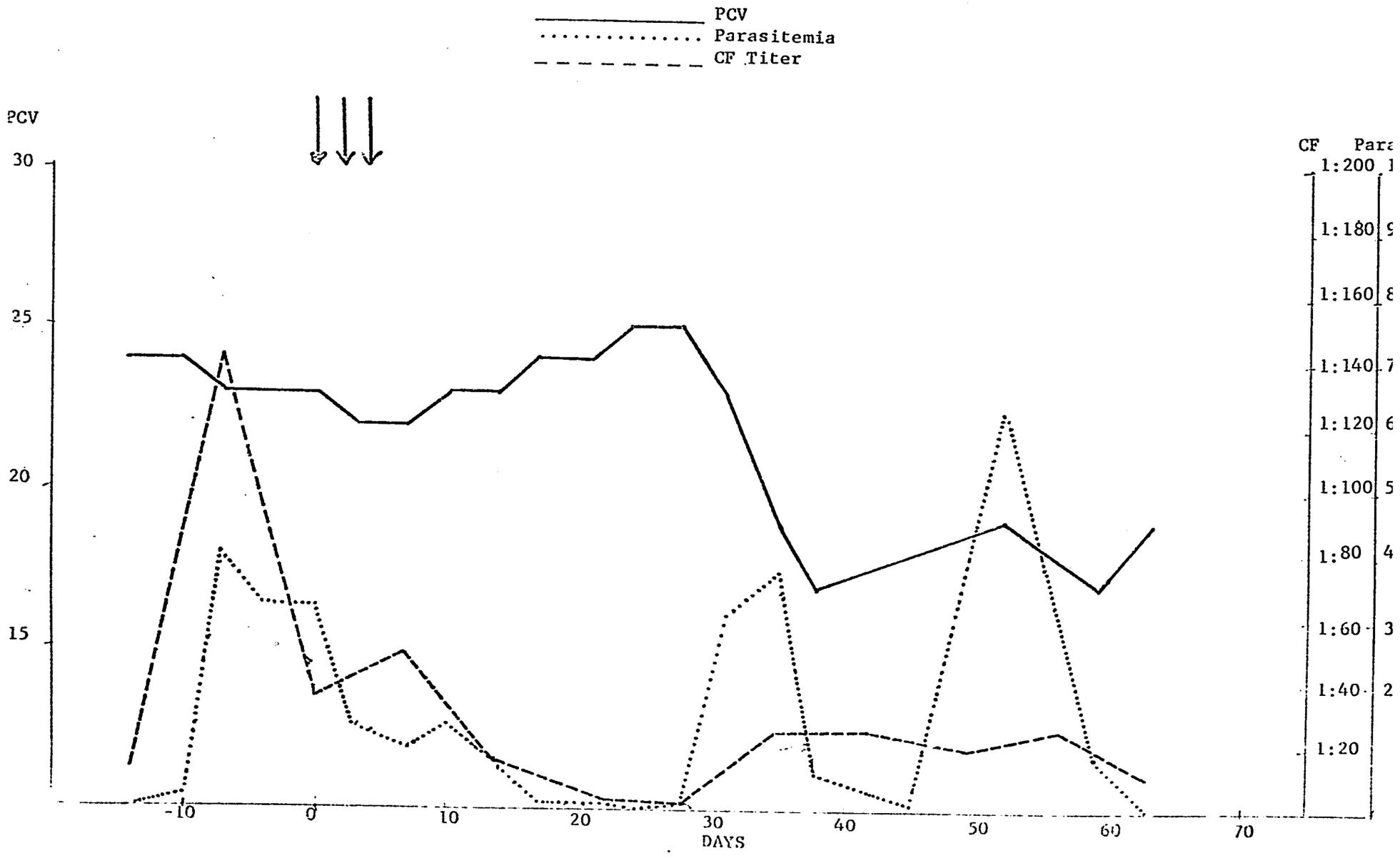


Figure 6

Group VI

5 Splenectomized Calves Treated 3 Times at 48 Hour Intervals With:
356C61 - 5 or 10 mg/kg

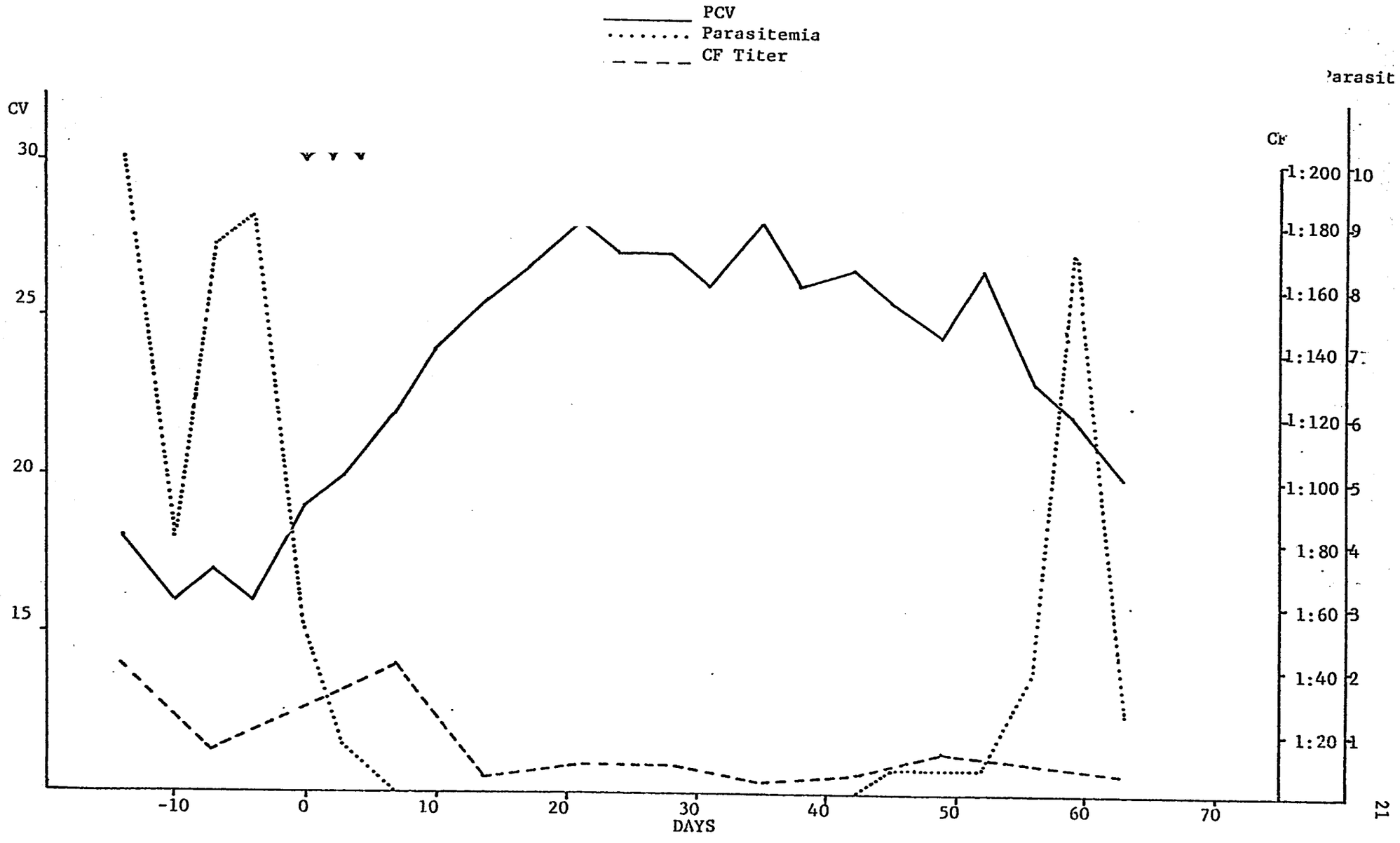


Figure 7

Group VII

3 Splenectomized Calves Treated 3 Times at 48 or 24 Hour Intervals With:
Oxytetracycline - 11 or 22 mg/kg

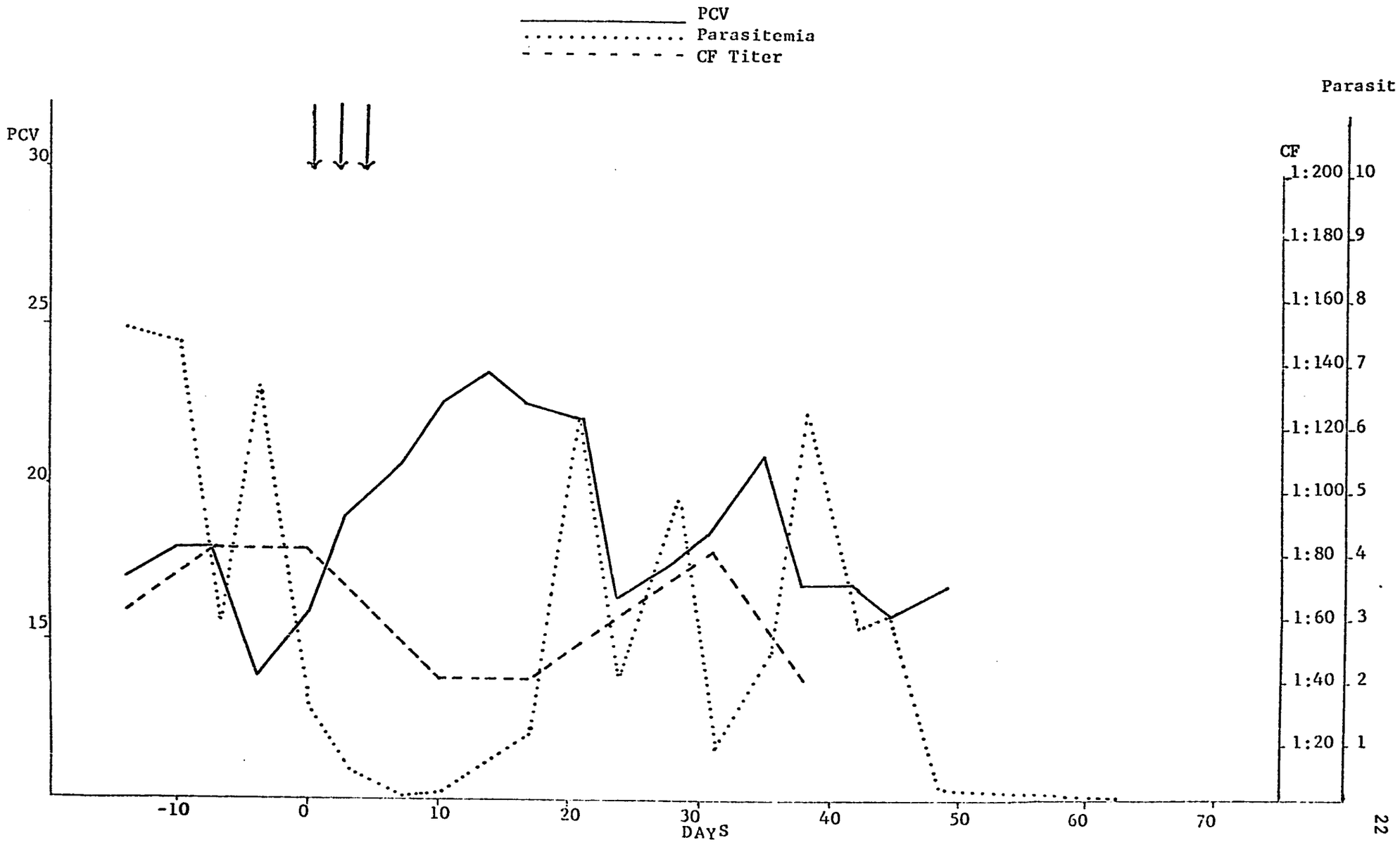


Figure 8

Group VIII

4 Splenectomized Calves Treated 3 Times at 48 Hour Intervals With:
4A65 - 2 mg/kg

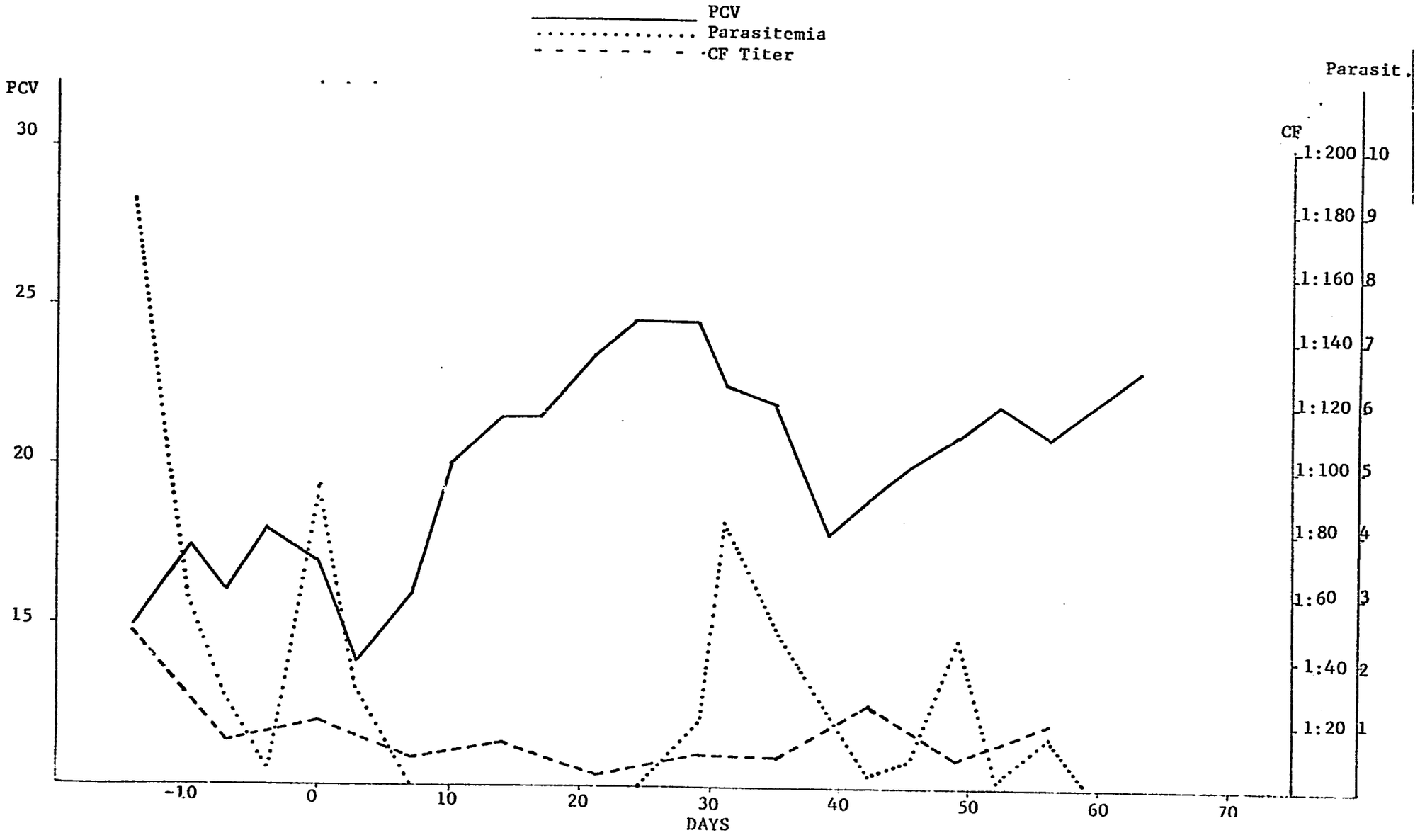


Figure 9

Group IX

3 Splenectomized Calves Treated at 72 Hour Intervals With:
356C61 - 5 mg kg

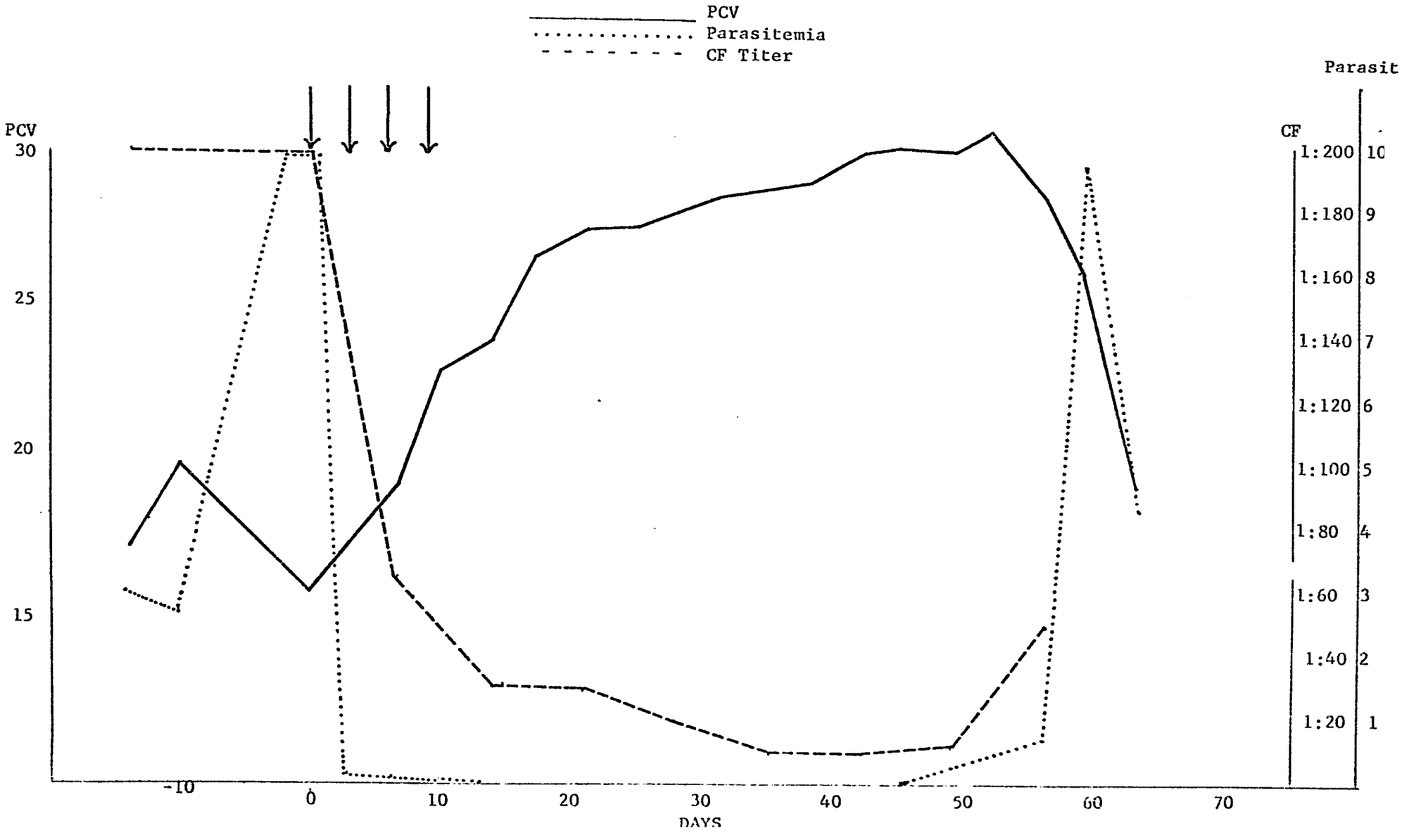


Figure 10

Group X

3 Splenectomized Calves Treated 3 Times at 7 Day Intervals With:
356C61 - 5 mg kg

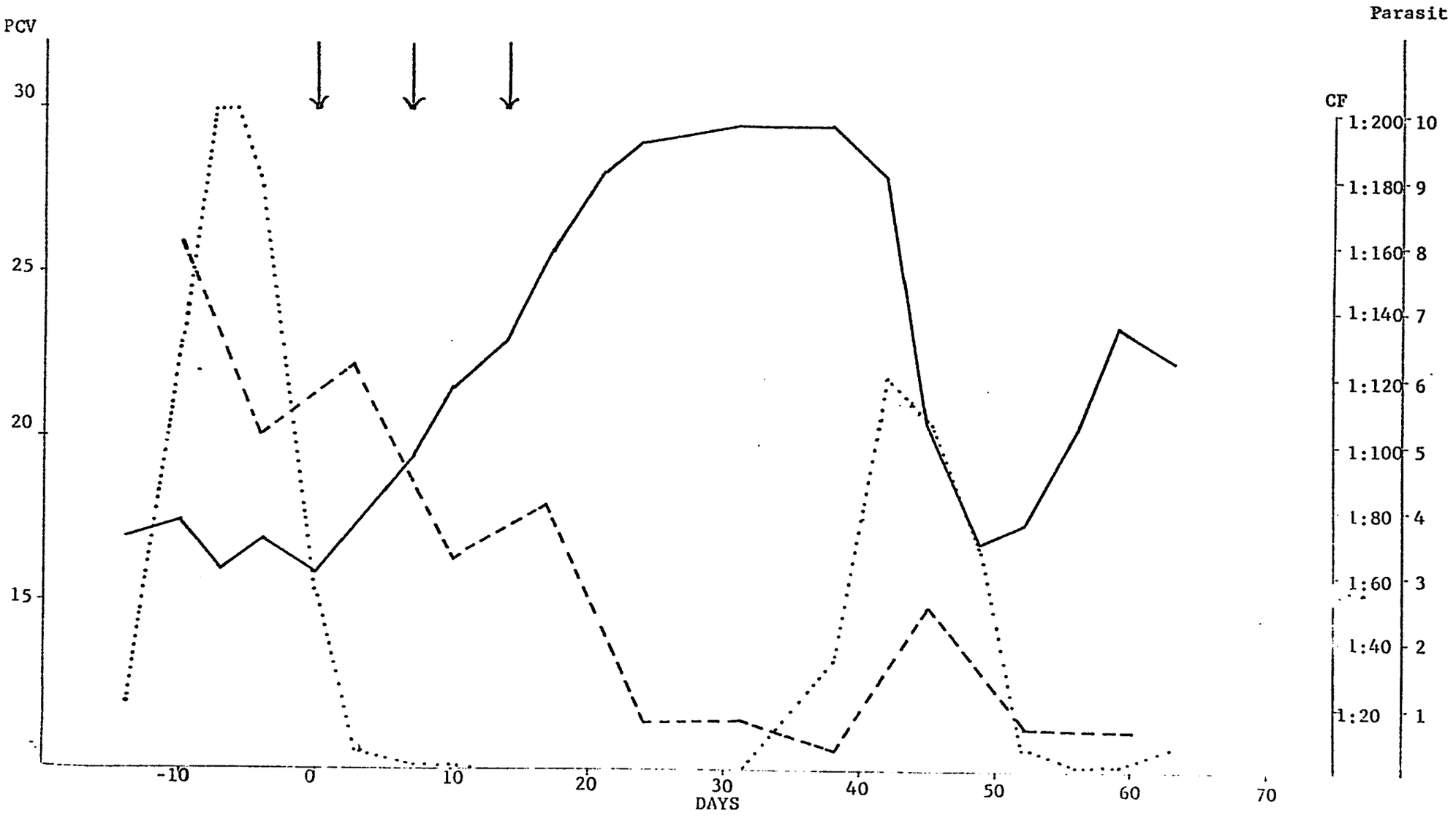
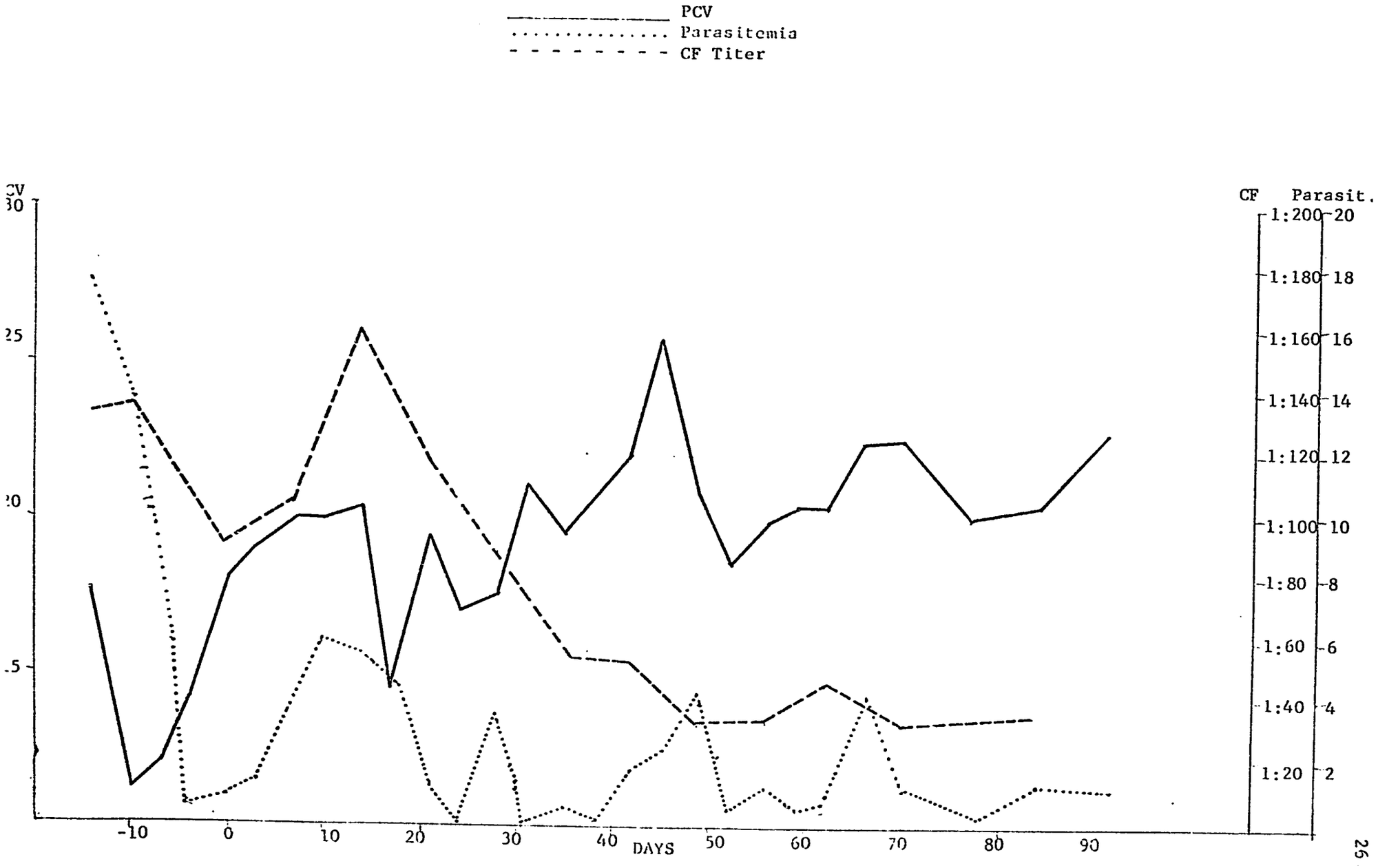


Figure 11

Non-treated Splenectomized Controls - 3 Weeks After Peak Parasitemia
Extending 80 days with No Treatment



Anaplasmosis Treatment Part II

The success in apparently eliminating anaplasmosis infections in splenectomized calves suggested the need to repeat this study using intact calves. In view of the natural resistance of young intact calves it was decided to use the lower level of treatment needed to free splenectomized animals.

A total of 11 calves of Holstein type, averaging approximately 6 months of age, were experimentally infected with virulent Anaplasma by the subcutaneous inoculation of 5 ml whole blood showing a 27% PCV and 1.5% parasitemia. Four of these calves were treated 14 days after exposure with 11 mg/kg oxytetracycline I.V. and 4 were treated 21 days after exposure in the same manner. The 3 remaining were untreated controls. Anaplasmosis developed in all animals. The incubation period and severity of infection was essentially the same in all groups. An average incubation time of 10 days was recorded for each group.

These calves were divided a second time into 3 groups so that treated and non-treated calves would be equally distributed, and then 100 days after initial exposure, treatment schedules were begun to eliminate infection. Four calves of Group 1, were treated with 5 mg/kg 356C61 and 11 mg/kg oxytetracycline, mixed in PSS and injected I.V. 3 times at 48-hour intervals. Four calves, comprising Group 2, were treated with 5 mg/kg 356C61 mixed in PSS and administered I.V. 3 times at 48-hour intervals. The 3rd group consisting of 3 calves remained as untreated controls.

Blood samples were taken from all animals twice weekly for the determination of PCV, CF, and parasitemia. All 11 calves were splenectomized either at 69 or 70 days after the last treatment to determine if in that time the infectious organism had been eliminated. Previous studies have shown that latent infections of Anaplasma, as well as other hemoparasite infections, will reappear following splenectomy.

Results of this trial are recorded in Table 2. Consistent with previous results the combined treatment of 356C61 and oxytetracycline was superior to 356C61 when given alone. Inconsistent with previous work with splenectomiz calves was a 50% relapse pattern observed in Group 1 following splenectomy.

Group 1 showed a favorable increase in PCV following treatment which proved, by paired comparisons, to be significant. Notwithstanding the treatment of these calves with both 356C61 and oxytetracycline, relapses occurred in 2 of 4 calves an average of 30 days after splenectomy.

In Group 2, 3 of 4 treated calves showed an Anaplasma relapse an average of 28 days following splenectomy. All 3 calves of the non-treated group showed an Anaplasma relapse an average of 10 days following splenectomy. In general, the non-treated calves relapsed earlier and showed a slightly more severe reaction than the 2 treated groups, however, these differences were not always significant.

Discussion and Conclusions:

More work with intact calves and cows is indicated. The assumption that infected splenectomized animals would constitute a more severe test of drug efficacy may be erroneous. It is also possible that splenectomy may have been done too early in these treated calves. Infectivity trials are usually conducted on splenectomized calves 90 days or longer after treatment.

These calves were splenectomized at about 70 days. There was obviously a residual Anaplasma infection in those animals showing relapse reactions.

Data more recently obtained on splenectomized calves does indicate that 356C61 alone is insufficient to eliminate infection. The only calves in this experiment that should have cleared up were those given the combination therapy.

Table 2

Treatment of Intact Calves to Eliminate Infection

	Group 1	Group 2	Group 3	Significanc
No. of Calves	4	4	3	
Age	9.2 mo.	9.5 mo.	9.3 mo.	NS
Treatment				
356C61	5 mg/kg	5 mg/kg	-	
Oxytetracycline	11 mg/kg	-	-	
No. of injections	3	3	-	
Pre-Treatment				
PCV	20.0	20.5	22.3	NS
Parasitemia	0.2	0.1	0.1	
Post Treatment Values Prior to Splenectomy				
PCV	26.8	26.0	23.7	NS
Significance of PCV Increase	P<0.05	NS	NS	
Parasitemia	0	0	0	
Post Splenectomy Response				
Relapses				
Number	2	3	0	
Average time	30 da.	28 da.	10 da.	NS
Avg. low PCV of Relapsing Cases	13.0	13.7	9.7	P<0.05
Avg. high Paras. of Relapsing Cases	28.0	18.7	33.7	NS

Anaplasmosis Treatment Part III

Second and Third Treatment of Calves Relapsing from a Previous Treatment

Treatment schedules, other than those containing both oxytetracycline and 356C61 given 3 times, have for the most part been unsuccessful in preventing an Anaplasma recrudescense in splenectomized calves. Four such calves having been treated by various means were treated a second time with 5 or 10 mg/kg 356C61 and 11 mg/kg oxytetracycline 3 times at 24 or 48 hour intervals as indicated in Table 3. A second treatment apparently eliminated infection in 3 of the 4 animals. Calf 245, however, showed a recrudescence 47 days after treatment.

This animal received a treatment schedule of 5 mg/kg 356C61 and 11 mg/kg oxytetracycline which in the past has been sufficient to eliminate infection. The 5 mg/kg level 356C61 when combined with oxytetracycline has ordinarily been adequate in splenectomized calves that had not been previously treated. The consistent elimination of Anaplasma by this treatment in the past does create questions relative to why this animal failed to respond favorably. The most obvious difference was the previous treatment with 356C61 and oxytetracycline an insufficient number of times to prevent a recrudescence, and the re-treatment of 245 with only a 5 mg/kg level 356C61, where as all others in this group responding favorably, had received the 10 mg level.

In addition to these 4 calves, observations were made on 7 other calves having a different history, but similar in that they were treated a second time after an Anaplasma relapse. Results are recorded in Table 4.

Calf 195 was first treated as an intact calf, carrying a latent Anaplasma infection, with 5 mg/kg 356C61 and 11 mg/kg oxytetracycline 2 times at a 4 day interval. Splenectomy was performed 70 days later, and after 12 days an Anaplasma parasitemia was noted. A second series of treatments consisting of 5 mg/kg 356C61 and 11 mg/kg oxytetracycline was give 3 times at 24 hour intervals. Treatment resulted in a marked increase in PCV 2 weeks after treatment but was followed by a relapse 42 days after treatment.

Calf 222 was first treated as an intact, Anaplasma infected calf with 5 mg/kg 356C61, 3 times at 48-hour intervals. Splenectomy was performed 69 days after treatment, and 56 days later an Anaplasma parasitemia was noted. A second series of treatments consisting of 10 mg/kg 356C61 and 11 mg/kg oxytetracycline were given 3 times at 48-hour intervals. A marked increase occurred in PCV 2 weeks after treatment and no evidence of relapse or Anaplasma recrudescence was observed.

Calf 225 was first treated as an intact, Anaplasma infected calf with 5 mg/kg 356C61 and 11 mg/kg oxytetracycline, 3 times at 48-hour intervals. Other animals in this group received treatment schedules initially which were inadequate to eliminate infection. This was not the case with Calf 225. Splenectomy was performed 69 days after treatment and 49 days later an Anaplasma parasitemia was detected. No good explanation is available for this failure. A second series of treatments consisting of 10 mg/kg 356C61 and 11 mg/kg oxytetracycline was given 3 times at 48-hour intervals. A marked increase in PCV occurred 2 weeks after treatment but was followed by an Anaplasma relapse 69 days later.

Calf 227 was first treated as an intact, Anaplasma infected calf with 5 mg/kg 356C61, 3 times at 48-hour intervals. Splenectomy was performed 70 days after treatment and 13 days later an Anaplasma parasitemia was noted, A series of 3 treatments with oxytetracycline, consisting of 22 mg/kg given at 48-hour intervals was given soon after this relapse. This treatment was unsuccessful, a relapse occurring soon after the last treatment. Another series of treatment consisting of 10 mg/kg 356C61, and 11 mg/kg oxytetracycline was given 3 times at 48-hour intervals (as recorded on Table 4). A marked increase in PCV occurred 2 weeks after this treatment, but was followed in 45 days by an Anaplasma relapse.

Animals 219 and 220 were not treated as intact, Anaplasma infected calves. Splenectomy was performed at the same approximate time as described for others of this group, and 10 days after surgery each animal developed a diagnostic Anaplasma parasitemia. Following this relapse each calf was treated 3 times with 2 mg/kg 4A65, I.V. at 48-hour intervals. This treatment was unsuccessful, a relapse occurring soon after the last treatment. Another series of treatments, recorded in Table 4, of 5 mg/kg 356C61 and 11 mg/kg oxytetracycline was given 3 times at 48-hour intervals. A marked increase in PCV occurred 2 weeks after treatment, and no evidence of Anaplasma relapse or recrudescence was seen.

Calf 226 was not treated as an intact, Anaplasma infected calf. An Anaplasma parasitemia occurred 10 days after splenectomy. At this time oxytetracycline treatment identical to that given calf 227 was administered, followed by the same rapid relapse. A final treatment, as recorded in Table 4, of 5 mg/kg 356C61 and 11 mg/kg oxytetracycline was given 3 times at 48-hour intervals. A marked increase in PCV occurred following treatment, but was followed by an Anaplasma relapse 45 days after the last treatment.

Conclusion:

The use of 356C61, 4A65, or oxytetracycline alone in the dose range reported will not prevent *Anaplasma* relapse in splenectomized calves. The use of 5 or 10 mg/kg 356C61 and 11 mg/kg oxytetracycline 3 times at 48 hour intervals gave sporadic results in previously treated splenectomized calves, whereas in previously non-treated splenectomized calves apparent elimination of *Anaplasma* infections was very consistent.

The reason for this treatment failure is not known, but one consistent feature is present in all cases which is suggestive of some relationship. In Table 4 calves 195, 225, 227, 226 all had received treatments containing oxytetracycline which were given in dose levels insufficient to eliminate the infection. These animals, when later treated with levels thought adequate to eliminate the *Anaplasma*, failed in every instance. Calves 222, 219, and 220 all had been treated with either 4A65 or 356C61 at levels inadequate to prevent relapse. These animals had not received oxytetracycline in the course of these treatments. These 3 calves, when treated a 2nd or 3rd time with either 5 or 10 mg/kg 356C61 and 11 mg/kg oxytetracycline, responded favorably and showed no signs of relapse. These results, while inconclusive at this time, suggest that a prior sub-effective level of oxytetracycline is related to failure to respond to the normally effective treatment schedule. This might lead to the assumption that a drug conditioned *Anaplasma* may develop when sub-lethal levels of oxytetracycline are introduced. We have had calves respond favorably to a 2nd treatment after exposure to oxytetracycline, so that a sub-effective level does not always result in a "conditioned" organism, but in every instance where treatment of splenectomized calves has failed with dose levels thought to be adequate, the treatment has been preceded by oxytetracycline treatment.

Table 3

Repeated Treatment of Splenectomized Calves

	1st Treatment				2nd Treatment			Data before 2nd Treatment		2 Week Post-Treatment		
Calves	356C61	Oxy. tet.	No. of Treat.	Relapse Time in days	356C61	Oxy. tet.	No. of Treat.	PCV	Paras.	PCV	Paras	Relapse Time Days
232	5mg	11mg	2	29 da.	10mg	11mg	3	23	1.0	26	0	No relapse
245	10mg	11mg	2	38 da.	5mg	11mg	3	16	7.0	28	0	47 da.
248	10mg	11mg	2	42 da.	10mg	11mg	3	19	0.1	28	0	No relapse
264	10mg	11mg	2	42 da.	10mg	11mg	3	17	0.8	27	0	No relapse
Average				37.8 da.				18.8	2.2	27.2	0	
S.D.				±6.1				±3.1	±3.2	±1.0		

Table 4

Re-Treatment of Calves Infected as Intact Animals and Later Splenectomized

	1st Treatment as an Intact Calf			Splenectomy		2nd or 3rd Treatment As A Splenectomized Calf			Values Before 2nd or 3rd Treatment		Values 2 weeks Post-Treatment		
Calves	356C61	Oxy.tet.	No. of Treat.	No. of days after Treatment	Relapse Time	356C61	Oxy.tet.	No. of Treat.	PCV	Paras.	PCV	Paras.	Relapse Time in days
195	5mg/kg	11mg/kg	2	70 da.	12 da.	5mg/kg	11mg/kg	3	20	0	26	0	42 da.
222	5mg/kg	-	3	69 da.	56 da.	10mg/kg	11mg/kg	3	16	1.5	22	0	No relapse
225	5mg/kg	11mg/kg	3	69 da.	49 da.	10mg/kg	11mg/kg	3	16	0.8	26	0	69 da.
227*	5mg/kg	-	3	70 da.	13 da.	10mg/kg	11mg/kg	3	16	13.0	26	0	45 da.
219**	-	-	-	70 da.	10 da.	5mg/kg	11mg/kg	3	14	5.0	27	0	No relapse
226*	-	-	-	70 da.	10 da.	5mg/kg	11mg/kg	3	19	0.3	28	0	42 da.
220*	-	-	-	70 da.	10 da.	5mg/kg	11mg/kg	3	18	0.5	29	0	No relapse
Average					23 da.				17	3.0	26		49.5 da.
S.D.					±20da.				± 2.1	±4.7	±2.2		±13.1 da.

*After splenectomy and relapse a schedule of 3 treatments with 22mg/kg oxytetracycline was given at 48 hour intervals. Relapse occurred rapidly and was then followed by treatment as indicated.

**After splenectomy and relapse a schedule of 3 treatments with 2mg/kg 4A65 was given at 48 hour intervals. Relapse occurred rapidly and was then followed by treatment as indicated.

Environmental Influence on the Course of Anaplasmosis in Splenectomized Calves

This study was started in the summer of 1969, and was completed during the summer of 1970. The incentive for pursuing this area of investigation was the differences noted in the severity of anaplasmosis occurring in warm or hot areas and those cases observed in cool or temperate zones. During early premunization studies, Schmidt observed that induced reactions were more severe during the summer, and hence, recommended premunization be carried out between November and March. Differences of varying degrees of significance were noted when Anaplasma infections occurring on the Bogota savannah were compared with those infections occurring at Palmira. Generally, infections were more severe in the warmer area.

In 1969, 3 splenectomized calves were maintained in air conditioned quarters during the summer, and 3 were kept outside. A mean difference of 17.5°F was recorded for the 2 groups. Anaplasmosis was induced in all 6 animals, by the same means, using uniform procedure throughout. The course of infection was followed carefully throughout a 75-day test period. The details and results are reported in the 1970 annual report. Generally, no significant differences were noted in either group to support the previous belief that ambient temperature is a factor in the severity of infection.

In the summer of 1970, part 2 of this study was done, using essentially the same experimental design, except that 4 splenectomized calves were used in each group instead of 3 as used the year before. Each group of 4 calves was approximately 7 months of age at the time they were exposed to anaplasmosis, and were all Holstein type, male, dairy calves.

Four calves were placed in the air conditioned room June 9, and on June 23 each of 8 calves was inoculated subcutaneously with 2 ml of blood from a frozen pool of known infected blood (August 13, 1969). Immediately following this inoculation each of the 8 calves was inoculated with 5 ml SPT* to suppress any possible exposure to eperythrozoon at the time of inoculation.

During the period June 16 to September 10, the mean temperature in the air conditioned room was 61.5 ± 2.8 whereas the average temperature outside was 85.5°F ± 11.0 (Table 5 and Figure 1). These temperatures were slightly lower than experienced last year, but the difference between the air conditioned room and outside temperatures was greater in 1970. In 1969 the average difference was 17.5°F whereas in 1970 this difference was 24.0°F.

During the actual time parasitemia was occurring, (July 29-Aug. 28) the average air conditioned temperature was 62.3°F (± 2.0) and the outside temperature 89.0°F ± 11.6; a 26.7°F difference.

Blood samples were taken twice a week for the entire period of the experiment, with PCVs, , CF, and parasitemias being recorded for comparisons.

When feasilble results obtained in 1969 were then added to those of 1970 to give larger numbers for statistical comparisons.

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* Spirotripan Fuerte, Farbweake Hoechst, Frankfurt.

Results:

The results obtained in the 1970 season are tabulated and presented in Table 5, and figures 2, 3, and 4. Calf 259, one of the four exposed to summer heat in the barn, died of acute anaplasmosis. No deaths occurred among calves kept indoors. A slightly higher CF titer, a slightly more severe anemic response, and a slightly higher parasitemia were seen in the barn group but these differences failed to reach significance. The average weight gains were greater among the barn group, but this also failed to reach significance. The overall incubation time was longer in 1970, probably due to the inoculum, which had been frozen for almost a year when used. The incubation time was actually shorter in the air conditioned group, although this was of questionable significance.

The combined results of 1969-1970 are presented in Table 6, and figures 5 and 6. Once again no obvious significant differences could be seen between Anaplasma infections in calves maintained indoors at a mean temperature of 65.3°F and in calves kept in an outdoor barn with a mean temperature of 85.9°F.

Small differences were noted with a trend toward more severe reactions in the barn group. The significance of the 1 death in the barn group is impossible to evaluate with the number of animals being used.

Figures 5 and 6 plot group averages of PCV and parasitemia on the period of active parasitemia for each animal (1969 and 1970). Day 1 was an arbitrary date selected on the basis of the 1st diagnostic parasitemia, which in this instance was an 0.5% level. From this point each PCV and parasitemia was ploted on a twice a week basis for approximately 40 days. This approach eliminated the incubation time factor, and plotted entirely the active period of infection. Even though some fluxuations were noted, no significant differences were detected between the 2 groups.

Conclusions:

In splenectomized calves the severity of anaplasmosis, when artifically induced by the inoculation of infectious material, was not significantly influenced by ambient temperature.

Factors involved in the obvious differences observed in the severity of induced anaplasmosis should be determined, as they could influence the safety and value of vaccination programs based on premunization with either virulent or attenuated agents.

Table 5

Environmental Influence on the Severity of Anaplasmosis in 8 Splenectomized Calves - (1970)

	Barn	Air Conditioned	Significance
Mean Temperature June 16 - September 10, 1970	85.5 ± 11.0	61.5 ± 2.8	
Avg. Pre-Infection PCV	33	34	NS
Avg. Incubation Time	37da.	33da.	NS
Avg. High Parasitemia	23.8	20.2	NS
Avg. Low PCV	9.8	11.8	NS
Avg. High CF	1:190	1:95	NS
Avg. wt. gain (6-8-70 to 10-9-70)	48.3 lbs.	45.0 lbs.	NS
Deaths	1	0	

Table 6

Environmental Influence on the Severity of Anaplasmosis in 7 Splenectomized Calves 1969 - 1970

	Barn	Air Conditioned	Significance
Mean Temperature during test period.	85.9	65.3	
Pre-Infection PCV	32.1%	32.0%	NS
Incubation Time	30.0 da.	27.3 da.	NS
High Parasitemia	31.1%	28.4%	NS
Low PCV	9.8%	11.8%	NS
High CF	1:190	1:95	NS
Deaths	1	0	

Figure 12

1970 Mean Temperatures

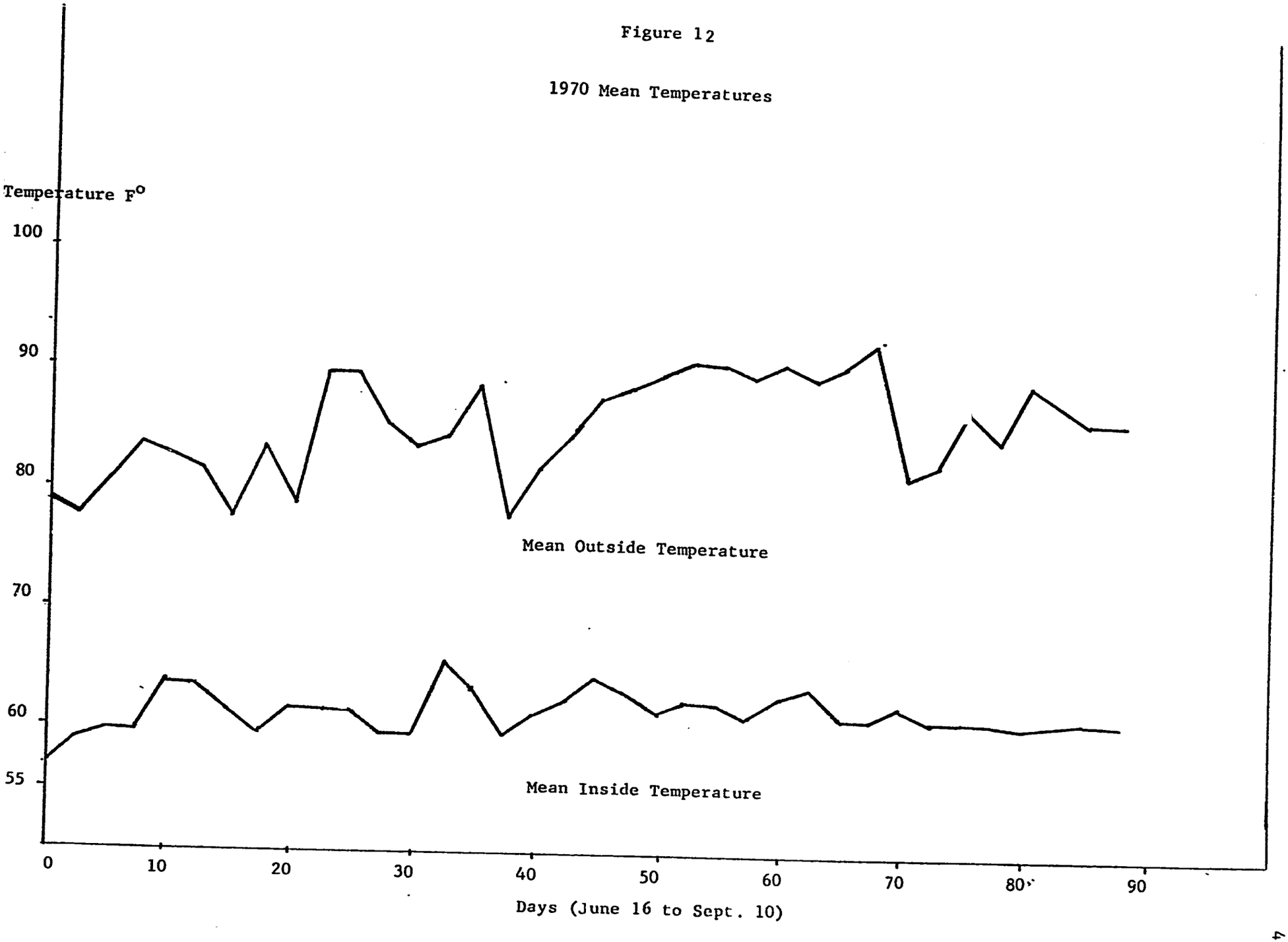


Figure 13

Packed Cell Volume
(1970)

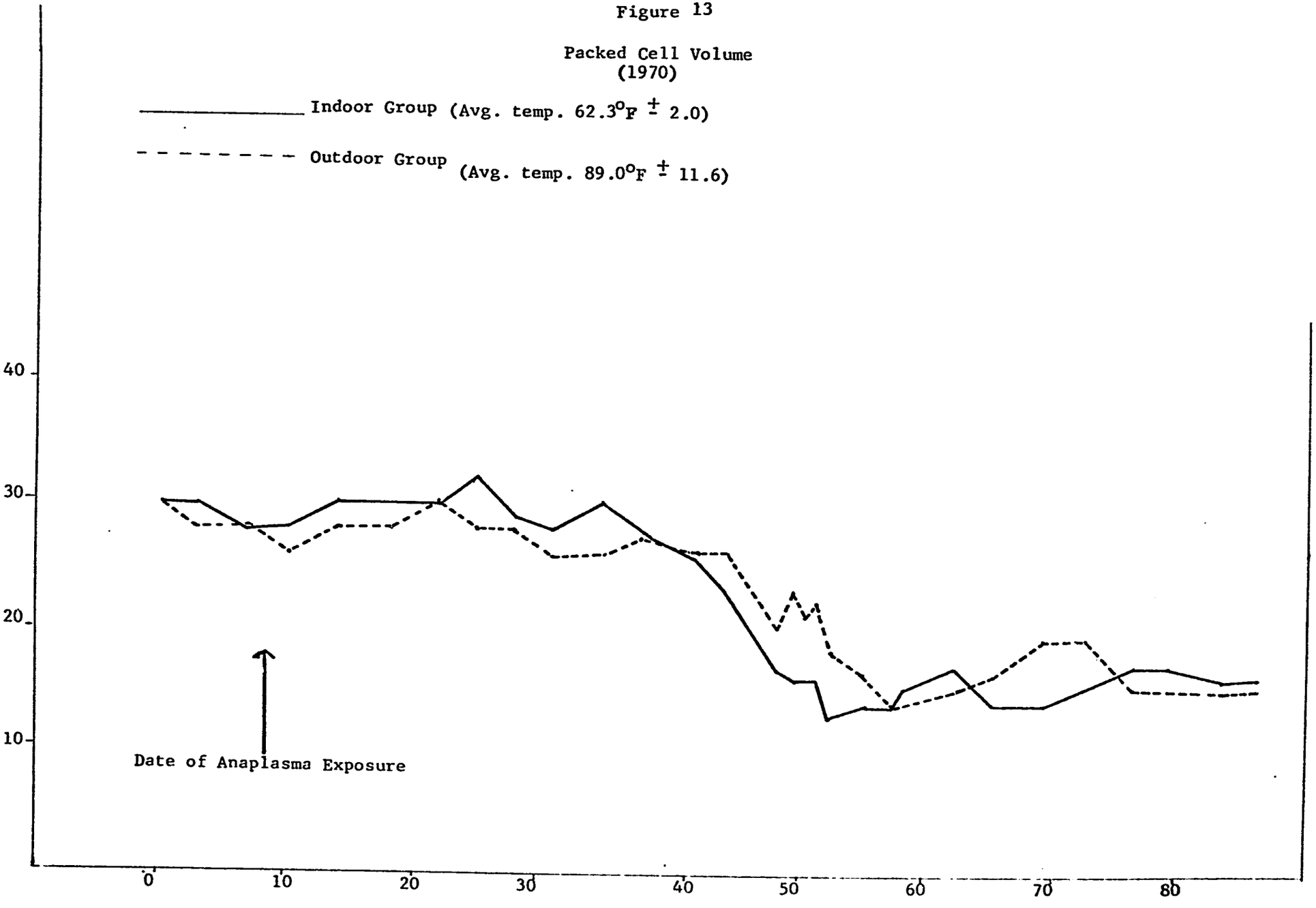
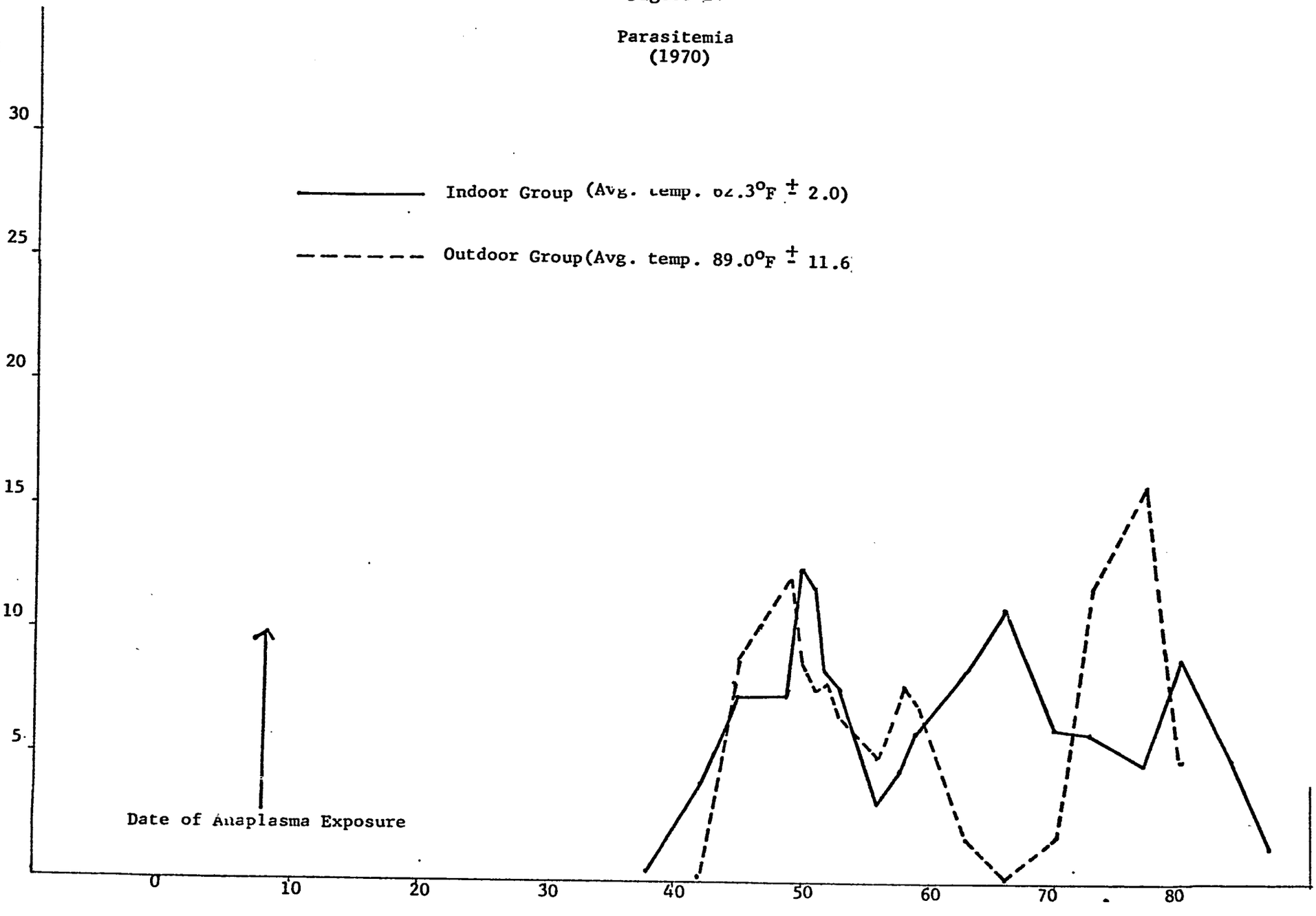


Figure 14

Parasitemia
(1970)



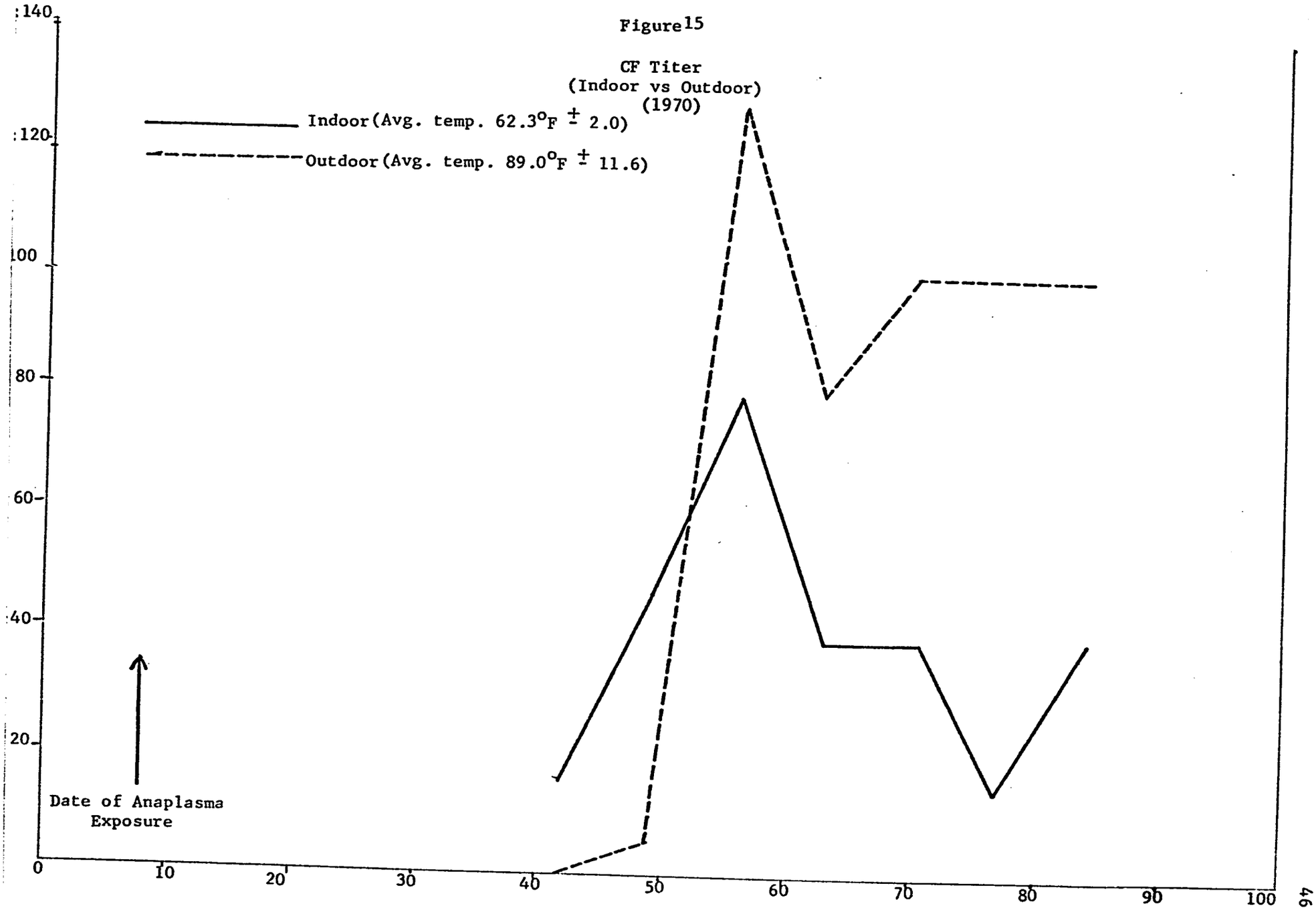


Figure 15
CF Titer
(Indoor vs Outdoor)
(1970)
Indoor (Avg. temp. 62.3°F ± 2.0)
Outdoor (Avg. temp. 89.0°F ± 11.6)
140
120
100
80
60
40
20
0
10
20
30
40
50
60
70
80
90
100
Date of Anaplasma
Exposure

Figure 16

Average Parasitemia Occurring in Calves
Maintained in an Air Conditioned Room
and Those in an Open Barn (1969 and 1970)

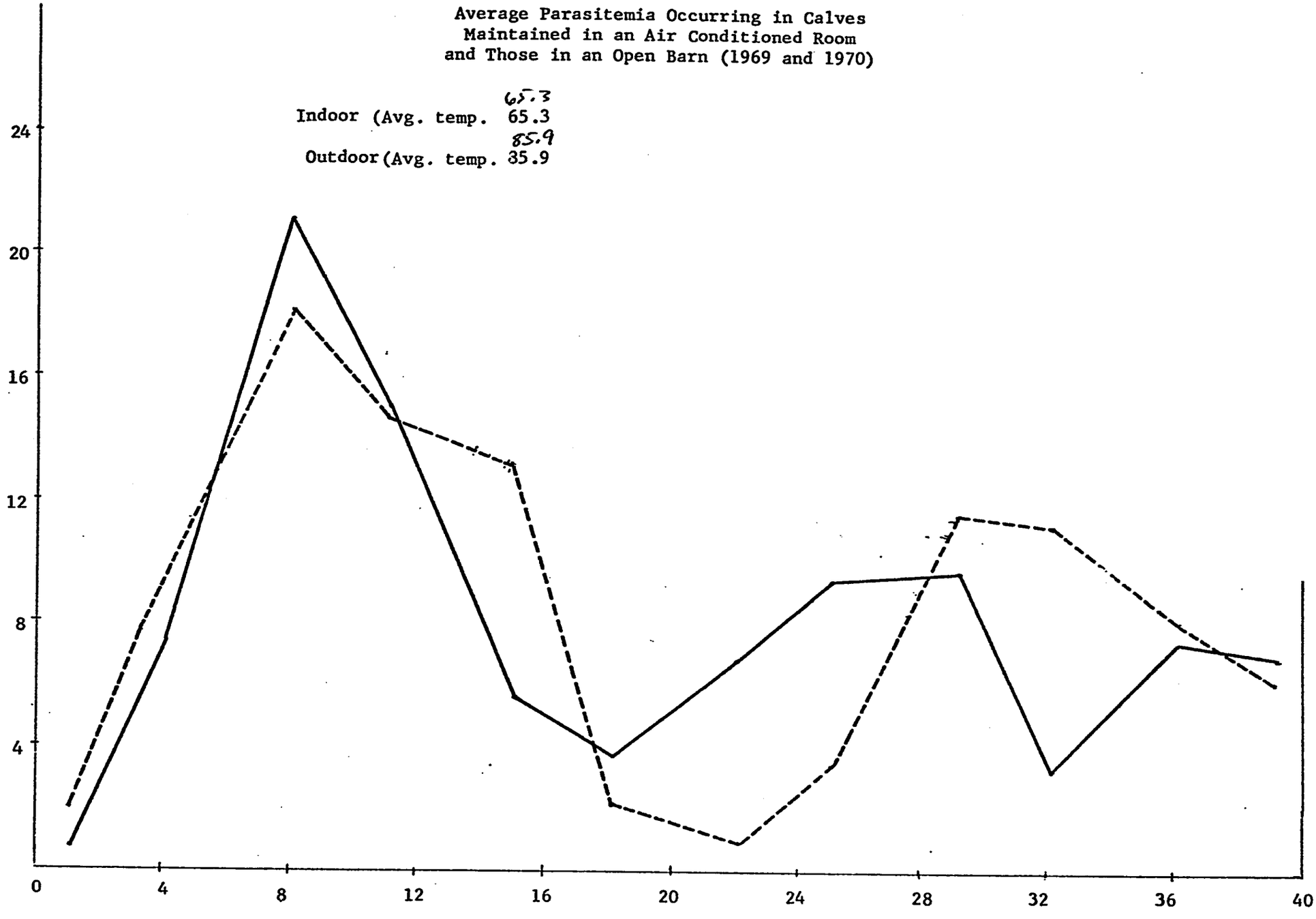
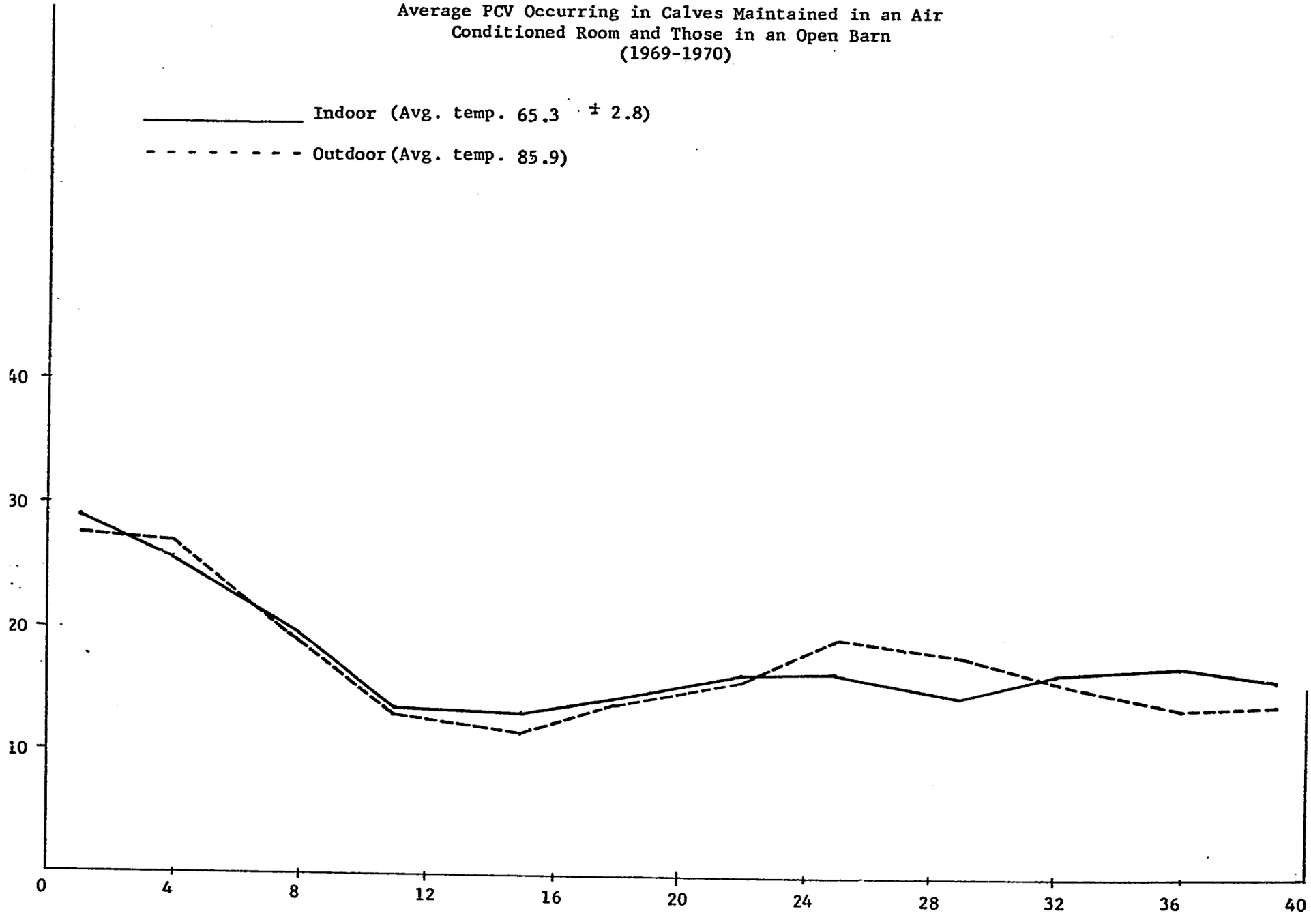


Figure 17

Average PCV Occurring in Calves Maintained in an Air Conditioned Room and Those in an Open Barn (1969-1970)



Effects of Freezing and Storage at -65°C on *Anaplasma* Infected Blood

There has long been the need to establish a bank or stock of known infective inocula for experimental purposes, in evaluating various immunological procedures. It is generally accepted that *Anaplasma* immunity is relative rather than absolute, and the challenge administered can become a very important factor in the final outcome. An animal which might be immune to minimal field challenge could develop anaplasmosis and succumb to the infection with a sufficiently heavy challenge. It is difficult to assess the infectivity of a given blood sample. Attempts to describe such inoculums are dependent on evaluating the parasitemia, CF titer, PCV, and in determining if the parasitemia is ascending or descending. These methods are not precise, and have never been adequately evaluated or correlated to infectivity by actual titrations. It would be advantageous to have a standard inoculum available that could be depended upon to have the same number of infective units each time the need occurred.

In addition to the desirability of having standard challenge material, a program of premunization against anaplasmosis would be greatly enhanced if the vaccine material could be frozen and maintained in quantity over a long period of time. The greatest advantage of such an approach would probably be safety factors, since known healthy donor cattle held in isolation and quarantine could be used, and hence prevent the possibility of promiscuous transmission of other blood born cattle diseases. If such an inoculum was sufficiently virulent as to require treatment, a known incubation time would be most helpful in establishing the optimum time for treatment.

The inoculation of bovine blood, for the purpose of premunization, into other cattle will always be accompanied by a risk of transmitting other bovine pathogens which might be non-apparent in the donor animal. If a non-bovine source were used for premunizing material, this hazard could be reduced.

Materials and Methods:

A total of 41 ml citrated blood from calf 1, having a PCV of 19% and a parasitemia of 44%, was mixed with 82 ml blood from calf 2, 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.

The infectivity of this material has been checked in 19 calves up to 486 days after freezing by inoculating subcutaneously the equivalent of 1 ml undiluted blood. For convenience in comparing the influence of storage, these calves were combined into 3 groups. Group 1 consisted of 5 splenectomized calves inoculated at 8, 36, 70, 182, and 266 days after freezing date. Group 2 consisted of 8 splenectomized calves, each inoculated 313 days after freezing. Group 3 consisted of 6 adult cows averaging 10 years or over in age. Group 3, while not entirely compatible with splenectomized calves, does contribute, since in the field this group will more likely be encountered. Roby, et. al., has reported that splenectomized calves and non-splenectomized cows were found to be similarily susceptible to Anaplasma infected blood.*

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* Roby, T.O., Gates, D.W., and Mott, L.O.: Am. J. Vet. Res. 22 (Nov., 1961) p. 982.

The average incubation time, low PCV, high CF, and the high parasitemia were recorded for each group.

Standard deviations were expressed for each group, as well as an analysis of variance to determine possible significant differences.

A blood collection was made from deer 165 at a time when the PCV was 22% and the parasitemia 25%. In this instance the blood was mixed in equal parts of 4 Molar DMSO (7.8%) in 0.85% NaCl, after which 1.4 ml aliquots were frozen and stored at -65°C.

The infectivity of this material has been checked in 5 splenectomized calves by inoculating subcutaneously the equivalent of 1 ml undiluted blood. These calves were combined as group 4, and were inoculated 16, 35, 68, 112, and 196 days after freezing date. Comparisons as to incubation time, low PCV, high CF, and high parasitemia were made with Group 1 to determine the similarities of the disease produced by infected blood of a non-bovine source.

Results:

Results for Group I, II, and III are tabulated in Table 7. The wide differences in inoculation time in Group 1 is adequately reflected in the large standard deviation. No obvious differences were apparent during this time in the incubation period, or other factors which would suggest a change in infectivity of the frozen inoculum associated with time. This is reflected by the relatively low standard deviations of the incubation time, low PCV, and high parasitemia. In every instance, except the average storage time, the standard deviations were less in Group I than Group II, even though Group II animals were all inoculated on the same day. The variations in Group I can therefore be expected to reflect animal differences rather than a relationship to storage time.

There are no significant differences between Groups I and II, even though animals in Group I were inoculated on an average 112 days after freezing, and Group II were inoculated 313 days after freezing. Similar results were observed in Group III, which were inoculated 486 days after freezing. In this group, however, a significantly lower parasitemia occurred when compared to Groups I and II. The data presently available is insufficient to determine if this represents an animal difference or is related to storage time. It is probably the former. Notwithstanding the lack of significant differences in these groups, an unmistakable trend appears, which seems related to storage time. In every instance the incubation times are slightly longer in Groups II and III, and the low PCVs are progressively higher. The significance of these observations can only be made following the inoculation of other calves at more prolonged storage times. This work is being continued.

A comparison between infections induced by frozen inoculums of bovine and deer origins are presented in Table 8. Infections resulting from the inoculation of Anaplasma infected deer blood, frozen and stored similar to the methods used for bovine blood, showed only minor differences. The storage times varied widely in each group, and for the time observed, no differences could be related to storage time. The incubation time, low PCV, and high CF titer were not significantly different between Groups I and IV. A significant increase in parasitemia was noted in Group IV.

This data does not suggest that A. marginale, as found in deer blood, is any less infective or invasive, following freezing and storage, than bovine blood.

Conclusion:

Storage of infected blood of bovine origin is practical for up to 486 days with little or no loss in infectivity as measured by factors recorded in this test. Such a blood bank could be used as a source of standard inoculum, or for premunization purposes.

Storage of *Anaplasma* infected blood of deer origin is equally as infectious and useful for up to an average of 85 days, producing infection at least as severe as produced by infected bovine blood. It is reasonable to suspect that continued studies will reveal that the storage qualities of such blood is comparable to bovine blood. A non-bovine host, as a source of infectious materials, offers distinct advantages when premunizing cattle as previously discussed.

Table 7

Influence of Storage at -65°C on
Anaplasma Infectivity

	No. of Animals	Average Storage Time	Average Incub. Time	Average Low PCV	Average High CF	Average High Paras.
Group I	5	112 da ± 108	32.8 ± 3.8	9.2 ± 1.5	1:160	19.8 ± 4.8
Group II	8	313 da ± 0	34.9 ± 6.5	10.5 ± 4.1	1:130	22.0 ± 16.5
Group III	6	486 da ± 14	38.5 ± 4.0	12.3 ± 1.8	1:226	9.2* ± 2.0
Significance		NS	NS	NS	NS	$P<0.05$

Groups I and II - Splenectomized calves of comparable age.

Group III - Adult cattle with an average age of over 10 years.

NS - Not significant

* Significantly lower than values for Groups I and II.

Table 8

Relative Infectivity of *Anaplasma* Infected Cattle and Deer Blood Stored at -65°C.

	No. of Animals	Average Storage Time	Average Incub. Time	Average Low PCV	Average High CF	Average High Paras.
Group I (Bovine)	5	112 da ± 108	32.8 ± 3.8	9.2 ± 1.5	1:160	19.8
Group IV (Deer)	5	85 da. ± 72	35.8 ± 4.1	8.4 ± 1.8	1:242	47.4 ± 22.3
Significance		NS	NS	NS	NS	$P<0.05$

NS - Not significant

Premunition Experiments Comparing *A. marginale*, *A. marginale* (attenuated) and *A. centrale*

An experimental anaplasmosis vaccine was received early in August, 1970, in good condition from Diamond Laboratories, Des Moines, Iowa. This vaccine is a live, attenuated *Anaplasma marginale*. Past experience has indicated that proper shipping and handling of this vaccine is very critical. It has been attenuated by numerous serial passages through splenectomized sheep, and actually consists of frozen sheep blood.

A total of 15 cattle making up a service herd on the Veterinary Research Farm were each inoculated subcutaneously with 5 ml of this vaccine on August 18, 1970. There were 8 cows and 1 bull 4 years of age or older, and 6 calves less than 1 year of age. Of the 9 mature cattle, one died of undetermined causes 21 days after vaccination before any evidence of *Anaplasma* response had been detected. A second cow was treated 9-14-70 for foot rot, and on 10-8-70 aborted a bull calf. It does not seem likely that vaccination was involved in the abortion since at the time this occurred response to vaccination was minimal, being limited to a positive CF response. This animal showed a somewhat atypical *Anaplasma* response which may or may not have been influenced by the preceeding events. To be on the safe side data from this animal was not included in our characterization of vaccine response. Four additional calves of similar age were vaccinated 10-23-70 with the same lot of vaccine, the only difference being the added 2-month storage time at-65°C.

The producing laboratory statesthat such storage is permissible, and will not influence the infectivity. At the same time the service herd was vaccinated 7, Anaplasma susceptible, splenectomized calves were turned out with the herd to test for the possible natural transmission of the vaccine strain.

After the vector season, on December 1, 1970, the splenectomized calves were removed from the herd and replaced by 6 mature cows. These animals were exposed to anaplasmosis by the inoculation of 2 ml standard inoculum subcutaneously. This inoculum consisted of equal parts of 6% DMSO and infected blood (parasitemia 42%, PCV 21%) which had been frozen in August, 1969, and stored at -65°C until thawed for this use.

Virulent anaplasmosis was induced in 11 intact calves of comparable age by the subcutaneous inoculation of 5 ml freshly drawn blood from a calf showing a 1.5% Anaplasma parasitemia and a 25% PCV.

In addition to the vaccine strain and virulent Anaplasma a group of infections induced by Anaplasma centrale were also used for comparison. A. centrale does not occur in the U.S. but is used widely throughout Africa, Asia, and Australia as a vaccine to prevent anaplasmosis. The infections referred to were induced in 1964 in Kenya, using cattle of European breeding, mostly dairy types, and under conditions similar to those tests conducted in Texas. A total of 8 mature cattle were inoculated subcutaneously with 5 ml of freshly drawn blood from a known A. centrale carrier calf. A total of 6 intact calves, of approximately the same age as used in Texas, were similarily infected.

Observations of PCV, CF, and *Anaplasma* parasitemia were made on all animals following exposure. The incubation time was that period measured in days after exposure until diagnostic evidence of anaplasmosis occurred, as determined by either a 1% parasitemia or, at least, a 4+ CF response at the 1:5 serum dilution.

Results:

The response to *Anaplasma* infections produced by *A. marginale* (attenuated vaccine strain) *A. centrale*, and *A. marginale* (virulent) is summarized in Table 9.

Mature cattle responded to exposure to all three *Anaplasma* strains. Infections produced by inocula which had been frozen and stored at -65°C for an extended period of time were associated with significantly longer incubation times than observed in *A. centrale* where fresh blood was used as a source of infectious material. This was most noticeable in the mature cattle where *A. marginale* (virulent) and *A. marginale* (attenuated) each had an average 38-day incubation, whereas *A. centrale* had an average incubation time of 15 days. The frozen inocula used to infect calves with the vaccine strain resulted in an average incubation of 28 days, whereas the fresh inocula used for *A. centrale* and *A. marginale* (virulent) resulted in incubation times of 10.8 and 10.2 days respectively. This data strongly suggests that the inocula used was the primary factor influencing incubation time rather than the *Anaplasma* strain.

As characterized by PCV, the response to vaccine strains (*A. marginale*, (attenuated) and *A. centrale*) appeared to be somewhat milder than seen with the fully virulent organism. There were significant differences in preinfection

PCVs, so that the anemia due to infections is not fully indicated in the low PCV. For this reason, a relative value termed "percent of normal" was inserted in Table 9 to better evaluate the degree of reduction in PCV resulting from infections with these 3 anaplasms . It was noted that A. centrale and the vaccine strain produced almost the same percent reduction in PCV, whereas the virulent organism produced significantly lower relative PCV. The parasitemia, while higher in the virulent group, was not significantly so. No significant difference in CF titers was detected. The duration of anemia was determined as the number of days the PCV remained 75% or less of normal. Using this criterion A. marginale (virulent) was responsible for anemia persisting considerably longer than seen with either of the vaccine strains. No significant difference was observed between the 2 vaccine materials.

A similar pattern of animal response to the 3 Anaplasma strains tested was noticed in non-splenectomized calves, but with differences that were not so great, more variable, and hence, less significant.

The differences in incubation time primarily reflects the inoculum used and not strain differences.

A significant difference existed in pre-infection PCVs, again requiring the use of "percent of normal" PCV values to evaluate the degree of anemia. The actually average low PCVs in the 3 groups were significantly different, but the relative PCV when compared to pre-infection norms failed to show significance even though lower values were observed with A. marginale (virulent). There were no significant differences between the parasitemia and CF titer.

Of unexpected occurrance was the persistence of anemia, of unusually long duration, in the group receiving A. marginale (virulent). The average duration of anemia, of 48.5 days, in the groups receiving A. marginale (virulent), was significantly longer than seen in either of the 2 vaccine groups.

The Diamond Laboratory vaccine failed to infect 3 of 10 calves inoculated. The values previously referred to and recorded in table 9 were calculated only on those reacting calves. Of considerable significance was the absolute failure of 3 calves to react when inoculated with the vaccine. This represents a 30% failure, which if occurring in field vaccination, would result in 30% being susceptible to later challenge exposure, with probably no resistance. This failure of intact calves to react to the vaccine strain has been observed in the past and is recorded in the 1967-69 progress report, where it was noted that 8 of 15 calves vaccinated on the Bogota savannah failed to react. This failure at Bogota was thought to be related to environmental factors, but these factors were absent during the summer and fall in Texas. It seems more likely that the frozen inoculum, consisting of sheep blood, is of such low infectivity that it cannot overcome the innate resistance of the young intact calf.

In comparison with A. centrale, the Diamond vaccine strain appears to be equally as mild. Previous studies (1967-69 report) indicate that the premunition with the Diamond vaccine will produce a solid resistance against a Texas strain of A. marginale (virulent) challenge, which is superior to the more relative protection resulting from premunition

with A. centrale. Field challenge at Monteria of calves and yearlings vaccinated with the Diamond vaccine resulted in a reaction which resembles the response seen in A. marginale challenge of A. centrale carriers. Adult cows, receiving the vaccine strain, reacted severely in 2 instances where PCVs of 11% and 13% were recorded. The animals recovered promptly, and did not show clinical signs of illness, but such low values are cause for concern. If heavily parasitized cattle, or animals with a low PCV from other causes were vaccinated, and the same relative response occurred, some death losses may have been encountered.

During the period of August to December when susceptible, splenectomized calves were in close contact with cattle reacting with the vaccine strain, no evidence of transmission was detected.

Periodic blood samples are being taken to measure the persistence of the infection with the attenuated A. marginale. Transmission and serologic studies have indicated the continued presence of the organism 7 months after vaccination.

Figure 1 indicates the average PCV and parasitemia response in mature cattle vaccinated with the attenuated organism and the time required for a return to normal.

The vaccine, while a marked improvement over premunition with virulent Anaplasma, must be used with some degree of caution, and preparation made to treat over-reacting old cows. Subsequent checks need to be made on young calves to be sure a "take" has occurred or else use the virulent organism for premunition. In calves less than 10 months of age the virulent organism does not result in overly severe reactions, and is capable of infecting 100% of the calves exposed. It is thought that the

use of fresh inocula from A. marginale (attenuated) carriers might improve the percentage of "takes", with subsequent premunition and immunity.

Table 9

Relative Animal Response to Premunition with *A. centrale*, an Attenuated and a Virulent *A. marginale*.

	No. of Animals	Pre-Inf. PCV	Incubation Time Days	Low PCV	% of Normal Low PCV	High Paras.	High CF	Duration of Anemia Days	Number Showing No Response	Deaths
MATURE CATTLE										
Diamond Vaccine	7	30.4	38.3	19.7	65	6.2	1:320	15.7	0	0
A. centrale	8	35.8	15	[illegible].5	66	5.1	1:207	12.0	0	0
Virulent *A. marginale*	6	26	38.3	12.3	47	9.3	1:180	24.3	0	0
Significance		P<0.01	P<0.01	P<0.01	P<0.05	NS	NS	P<0.01	-	-
DRS:		4.6	14.8	6.2	15.5			7.7		
CALVES										
Diamond Vaccine	10[1]	31.0	28.3	20	65	2.0	1:260	10.4	3	0
A. centrale	6	35.0	10.8	23.5	67	5.6	1:320	7.7	0	0
Virulent *A. marginale*	11	29.6	10.2	17.1	58	0.6	1:250	48.5	0	0
Significance		P<0.01	P<0.01	P<0.05	NS	NS	NS	P<0.01		
DRS:		3.5	2.8	5.8				23.3		

[1] Averages are calculated only on those 7 animals reacting.

NS: Not Significant

DRS: Difference Required for Significance.

Influence of Treatment with Oxytetracycline on *Anaplasma* Premunition of Intact Calves

The difficulty encountered with premunization of young intact calves with an attenuated organism as previously discussed in this and other reports (1969-1970) suggests that more reliable results might be obtained with a virulent organism. This method while appearing to be the one of choice for calves being moved into areas such as the North Coast of Colombia, does involve a hazard of over reaction and possible death.

In consideration of this possibility and in anticipation of a vaccine and control program to be instituted on the North Coast of Colombia it was decided that a preliminary trial would be conducted at Texas A&M to evaluate oxytetracycline as a drug to moderate the expected severe reaction in exposed calves.

A total of 12 Holstein-type calves, 5 to 6 months of age, were inoculated subcutaneously on day 0 with 5 ml whole citrated blood from calf 142 showing a 25% PCV and a 1.5% *A. marginale* parasitemia. Four calves were treated on day 14 with oxytetracycline (Liquamycin) at the rate of 11 mg/kg administered I.M. Four additional calves were treated on day 21 following the same procedure. Four calves remained as untreated controls.

Results:

The results are tabulated and illustrated in table 11 and figures 19, 20, and 21. The factors observed in table 11 were:

1.) Pre-infection PCV

2.) The incubation time expressed as days after exposure that diagnostic evidence of anaplasmosis was observed.

3.) The average low PCV associated with anaplasmosis.

4.) The low percent of normal, calculated for each animal to better detect the degree of anemia for each of the 3 groups, minimizing individual animal fluxuation.

5.) The days required to reach a low PCV after diagnostic evidence of anaplasmosis had occurred.

6.) Average high parasitemia occurring in the course of infection.

7.) The number of days during which the PCV was 75% or less than normal. This observation hopefully would tell us how rapidly the calf could throw off the effects of infection and return to a relatively normal condition.

8.) The average high CF titer.

There were no significant differences between the 3 groups in any of these catagories that would indicate a moderating effect on the course of infection when treatment with oxytetracycline was administered. In figure 19 and 20 following treatment, there appeared to be a slight drop in the average parasitemia and a slight increase in PCV, but if these effects were actually the result of treatment, they were minimal and very transient.

Discussion and Conclusions:

Evidence is lacking that oxytetracycline, when administered as indicated and at 14 or 21 days after anaplasmosis, resulted in any alteration of the course of infection. Oxytetracycline, when administered I.M. in the gluteus, did produce severe irritation and pain with many of the animals laying down, getting up, holding the affected leg up, etc.

Only 1 calf developed a low PCV which caused concern, and this was only 12%. A striking feature in this experiment was the prolonged period of time during which the anemia persisted. The added stress of *Babesia*, or possibly trypanosomes, to animals with a depressed PCV could well be responsible for a severe and, possibly, fatal anemia. In addition, observations of depressed PCVs are common in calves being moved to the North Coast even before they become exposed to hemotropic agents. For future work in Colombia, consideration should be given to the possible accumulative effects of these and possibly other factors all tending to reduce PCVs with particular attention being given to the eventual depletion of the erythropoietic systems capacity to replace lost erythrocytes.

Table 11

Influence of Treatment with Oxytetracycline on *Anaplasma* Premunition

	No. of Animals	Avg. Pre-Inf. PCV	Avg. Incubation Time in days	Avg. Low PCV	Low % of Normal PCV	Days required to reach low PCV after evidence of infection	Avg. High Parasitemia %	Duration of Anemia	Avg. High CF Titer
Treated 14 days after exposure	4	28.8	10	16.0	55.6	24.2	0.65	56	1/190
Treated 21 days after exposure	4	29.5	10	17.8	60.3	21.8	0.42	74	1/270
Not treated	4	29.5	10	16.0	54.2	23.5	0.58	77	1/270
		NS	NS	NS	NS	NS	NS	NS	NS

NS: Not significant

Figure 19

Treated with Oxytetracycline at 14 days

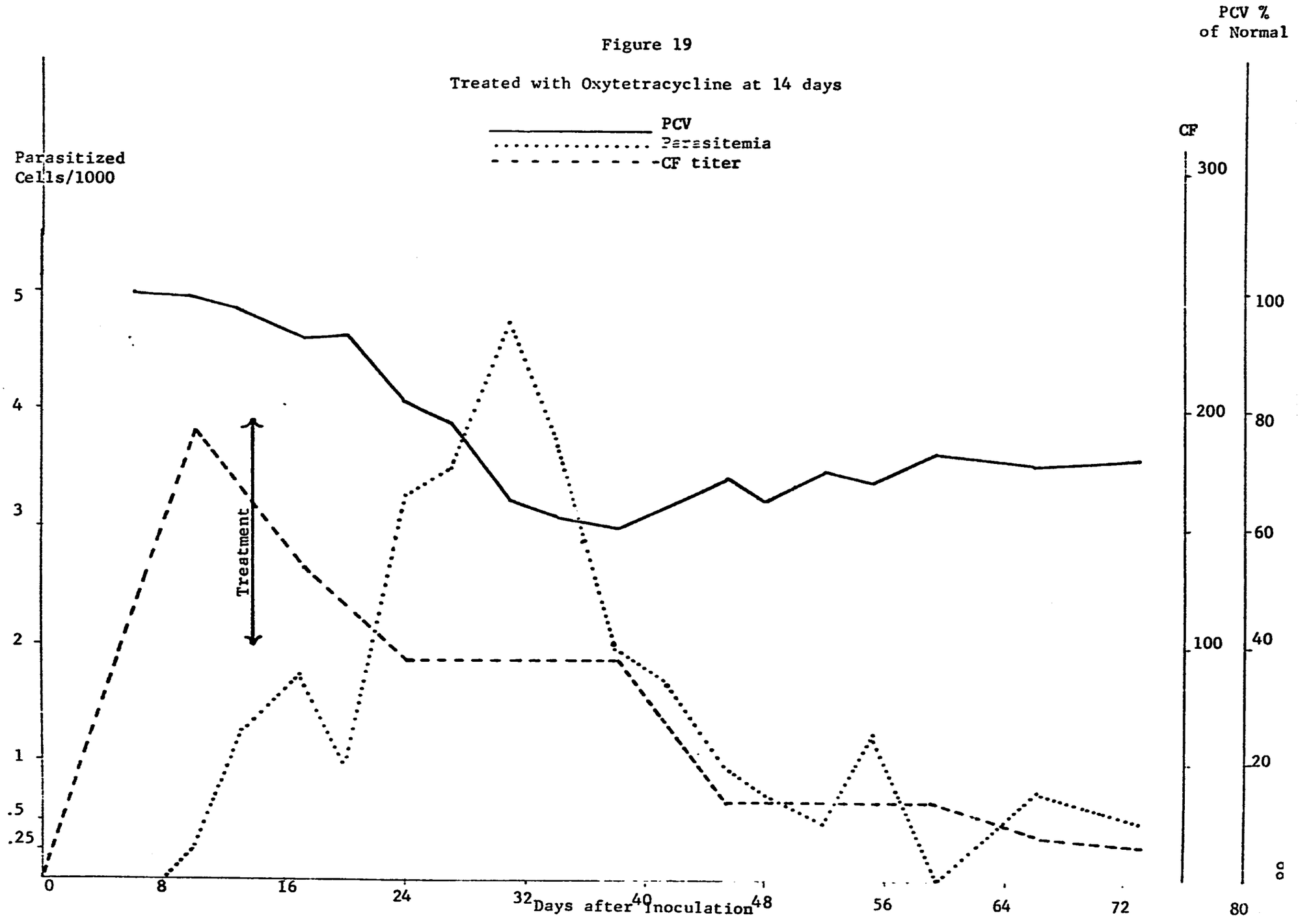


Figure 20

Treated with Oxytetracycline at 21 days

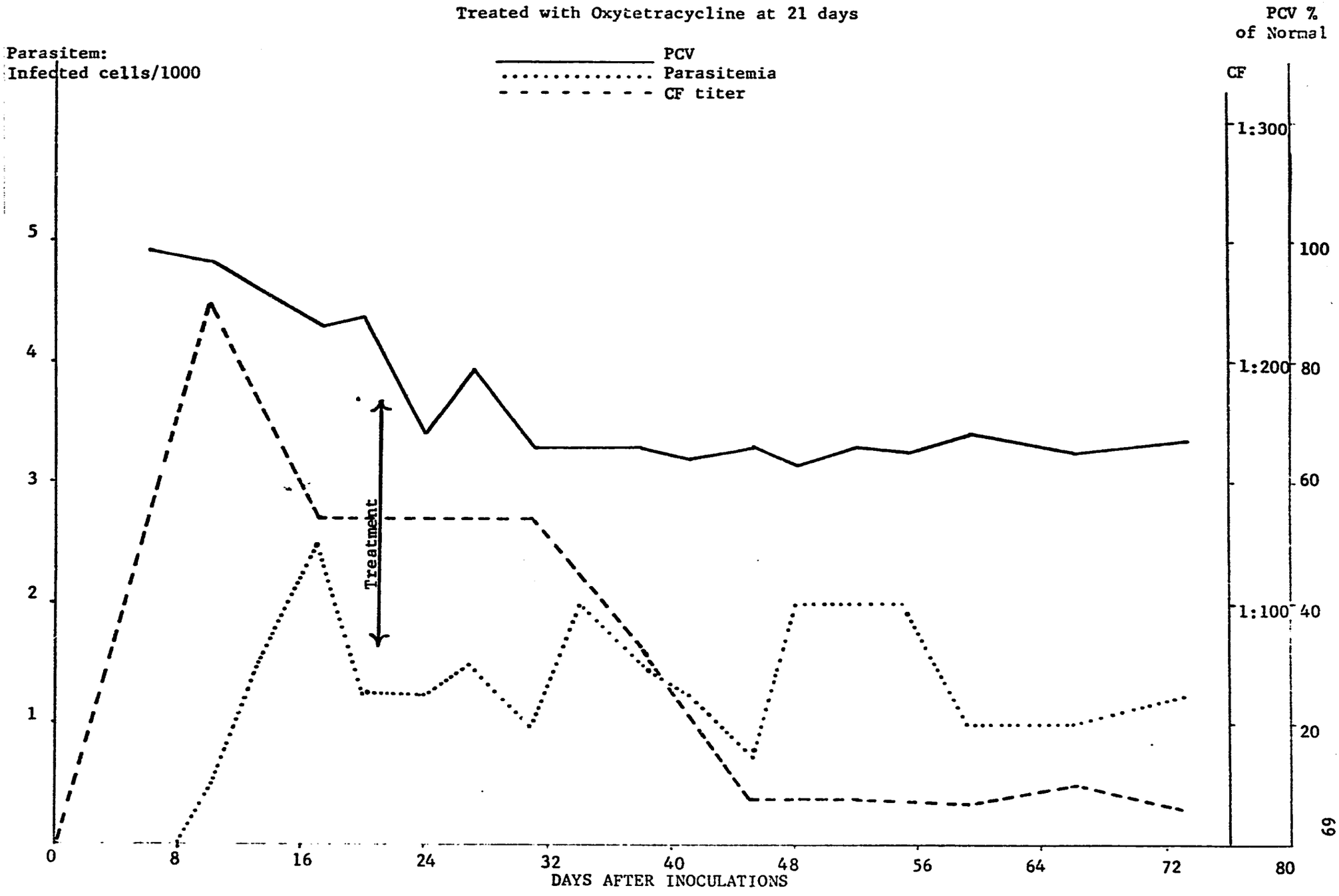
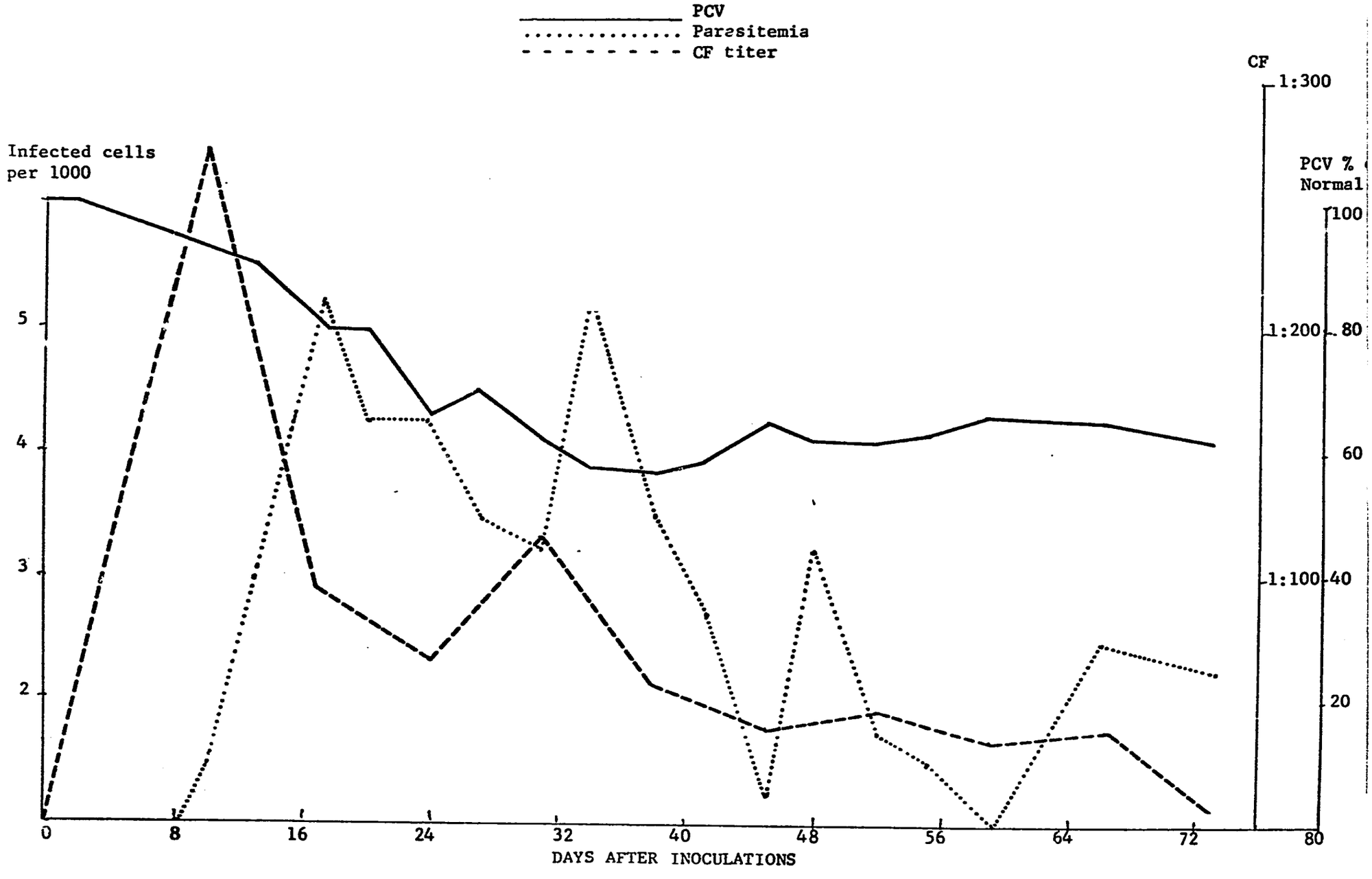


Figure 21

No Treatment



Relative Animal Response to A. marginale Relapse
and Re-Infection in Splenectomized Calves.

The seemingly paradoxical statement that to control anaplasmosis, anaplasmosis must be induced, frequently creates concern and doubt as to why such a course is necessary. In most instances recovery from a disease or the elimination of infection is followed by a period of immunity or at least a relative resistance to infection. It has generally been agreed, although not well documented, that the elimination of A. marginale infection is not followed by such a period of immunity, and that true immunity is dependent on the continued presence in the host of the causitive organism.

The development of a ki'led vaccine (Anaplaz) exposed this hypothesis to question, since it was apparently possible, by the injection of a killed antigens, to produce a relative resistance to anaplasmosis in the absence of active infection. An extension of this assumption creates questions as to whether or not there is a period following the removal of active infection when there is actually a residual or partial immunity that could be detected by a milder response to the second exposure or re-infection.

The development of a successful regime of drug therapy to remove active infection in a relatively short time provides us with an opportunity to better evaluate residual immunity by re-infecting animals at various times following drug therapy. To date, these experiments have included only animals that have been proved clean following drug therapy, by animal

inoculation and waiting for 120 days or longer for evidence of relapse, hence re-infection has been attempted at reasonably long intervals after elimination of infection. Now that a degree of confidence has been established in drug elimination of infection, re-infection can be accomplished at shorter intervals to measure residual immunity. Data in this report should therefore be considered preliminary in nature.

During therapy trials a number of treatment procedures were carried out which eliminated parasitemia for an extended period of 30-60 days, only to be followed by a relapse pattern. It is not known if during this negative phase the infectious organism is present in the blood, however evidence presented in the 1970 annual report (p.7) suggests that 12 days after treatment A.marginale could not be recovered, even though a relapsing infection occurred 49 days later in the same calf.

The elimination of parasitemia was generally accompanied by an increase in PCV, a reduction in CF titers, and evidence of complete recovery, only to be followed by a relapsing parasitemia, reduction in PCV and other evidence of acute anaplasmosis.

The object of this experiment was to determine:

1.) If re-infection of a known clean calf, previously infected, would result in a disease response different from the response to the initial infection.

2.) If animal response to relapsing infections, where an aparasitemic state had existed for 30 to 60 days, were significantly different from the response to the initial infection.

Methods:

A total of 5 splenectomized calves, infected with anaplasmosis were treated 3 times at 24 or 48 hour intervals with 356C61, 5 mg/kg and oxytetracycline, 11 mg/kg. Within a few days the parasitemia disappeared, a marked response in PCV occurred, and CF titer regressed. All calves were checked for infectivity 90 days or more after treatment by the injection of 200 ml of their blood into susceptible calves. The negative response in the check calves plus other evidence led to the conclusion that the treated calves were free of infection. On an average of 167 $\pm$ 56 days a second infection was induced by the inoculation of infected blood similar to that used initially. The severity of the first and second infection was characterized by the usual parameters, PCV, parasitemia, and CF response.

A total of 15 calves, infected with anaplasmosis, were treated with a variety of drugs and schedules unsuccessfully. In all of these animals, however, the parasitemia disappeared, and each presented evidence of having had the infection eliminated. In each instance relapse occurred. The 15 calves were selected on the basis of a relapsing infection from 30 to 64 days with an average of 46 $\pm$ 10 days. In these instances the severity of relapsing infections were compared to the severity of the initial infection by measuring the usual parameters of PCV, parasitemia, and CF response.

Results:

The results are summarized in table 10. The second infection produced on an average of 167 days after treatment proved to be equally as severe as

the initial infection with regards to PCV and parasitemia. Slightly higher PCV and slightly lower parasitemias were observed in the second infection, but these differences failed to reach significance. The average high CF titer encountered in the initial infection was 1:240 as compared to 1:46 in the second infection. This difference proved to be highly significant.

The severity of relapse infections occurring in 15 treated, splenectomized calves was slightly less than seen in the initial infection. The secondary relapse response was characterized by a higher PCV, a lower parasitemia, and a lower CF titer than seen in the initial infection. In every instance, these differences were statistically highly significant.

Conclusions:

With the exception of CF titers, no detectable, residual immunity in previously infected splenectomized calves could be detected 167 days after treatment. This observation once again confirms the need for active infection to maintain immunity. A need exists to re-infect recovered cattle at shorter intervals after treatment. This is further suggested by the milder secondary or relapse infection observed in animals showing a recrudescence following treatment. The interpretation of the significance of relapse infection is not clear, since there may have been multiplication of the causitive organism with a related undetected antibody response during the aparasitemic period, even though the evidence suggests a dormant or quiescent infection without active antibody production during this period. This assumption must be subject to question in the absence of more clear cut evidence, however.

Table 10

Relative Severity of *A. marginale* Relapse and Re-infection in Splenectomized Calves

Re-infection (5 Splenectomized Calves)

	Low PCV	High Paras.	High CF
Initial Response	9.6	31.2	1:240
Interval between last Parasitemia and Re-infection: 167 ± 56 days.			
Second Response	10.4	20.2	1:46
Significance	NS	NS	P<0.01

Relapse Infection (15 Splenectomized Calves)

Initial Response	10.1	30.2	1:270
Interval during which time no parasitemia seen: 43 ± 10 days.			
Secondary Response	13.9	12.6	1:88
Significance	P<0.01	P<0.01	P<0.01

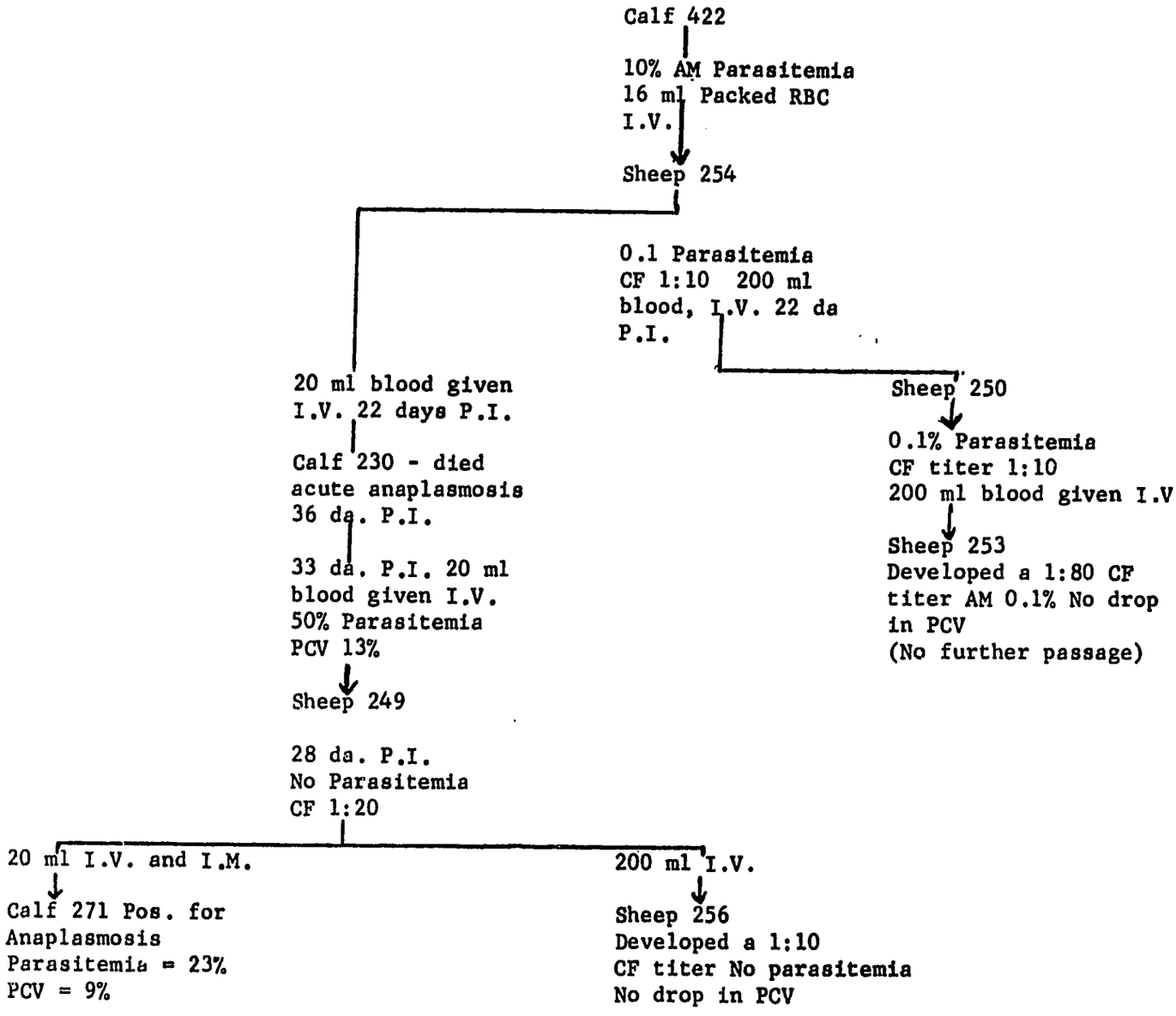
Sheep Passage

A series of experiments to pass Anaplasma marginale in sheep were conducted with varying degrees of success. Initially A. marginale from a bovine source was passed into sheep. The first passage in sheep was successful in that a specific CF response was noted along with a low level parasitemia. A. marginale was recovered on back passage to a splenectomized calf. Serial sheep to sheep passage was successful, but was not attempted beyond the 3rd passage. (See flow sheet).

Later the Diamond vaccine strain of A. marginale was received, and an attempt was made to produce a high parasitemia by rapid serial passage of infected blood. No difficulty was encountered in maintaining the organism, but in no instance did the parasitemia exceed 19%.

Sheep Passage

Flow Sheet



PI - Post Inoculation
IV - Intravenously
IM - Intramuscularly

Physical Characteristics of the Infectious Agent for Anaplasmosis

Numerous studies have been made during the past 10-15 years relative to the size, shape, location, and other physical characteristics of the infectious organism for anaplasmosis. The organism has been classified as a protozoa, a virus, and a rickettsia, without ever achieving the unanimity necessary for a satisfactory placement in any of these categories. It is probable that most authorities on this subject now favor the rickettsia classification. The organism has been reported to pass a 300 mu filter, but not a 220 mu filter. Numerous attempts have been made to separate the infectious organism from the red cell stroma for purposes such as preparing a more specific serologic antigen and in recent years to produce a safer antigen for vaccine purposes. Methods such as differential centrifugation, and sucrose gradients have been used with limited success, but for the most part these techniques are so involved that insufficient quantities of the purified antigens can be extracted for practical use.

In the belief that extracellular infectious particles exist in plasma, a few preliminary trials have been conducted to varify this and to obtain an idea concerning the degree of infectivity.

Calf 232, showing an 11% ascending parasitemia and a PCV of 20%, was used as a source of infecting inoculum. A total of 100 ml of blood was collected in citrate, and centrifuged 15 minutes at 2,000 RPM (5°C.). The plasma was removed carefully avoiding erythrocyte contamination. This plasma was centrifuged a 2nd time - 10 minutes at 2,000 RPM (5°C.) to remove any possible red cell contaminants. The clear plasma was then centrifuged at 5°C for 40 minutes at 18,000 RPM (in excess of 35,000 X G). The clear plasma was carefully collected using a syringe and needle and 10 ml injected I.V. into splenectomized calf 244.

Calf 244 developed a 1.5% parasitemia 30 days later which developed into a 55% parasitemia, a low PCV of 6.0%, and a CF titer of 1:320. In every respect the response was typical of anaplasmosis.

This experiment has been repeated on a number of occasions , and there seems little doubt that the infectious particle is present in cell free plasma. What is perhaps of equal interest, however, is the ability of the infectious particles to remain in plasma suspension even at 35,000 X G for 40 minutes. The significance of this finding relative to the collection of cell free antigens is being investigated.

A second part of this experiment involves a similar study of the infectious particles present in red blood cells. Calf 426 with an ascending secondary Anaplasma parasitemia of 19% and a PCV of 12% was used as a source of infected erythrocytes (RBC). The RBCs were washed 1 time in isotonic veronal buffer (pH 7.3) (VB) after discarding plasma. Approximately 12 ml of packed RBCs were mixed in equal parts with VB

and lysed by sonication 3 minutes at 80 watts. (Sonifer Cell Disruptor, Model, W185D, Heat Systems-Ultrasonics, Inc., Branson Sonic Power Co.). The RBCs were thoroughly lysed by this treatment. A total of 5 ml of this material was injected subcutaneously into splenectomized calf 500. Calf 500 first developed evidence of anaplasmosis 34 days afterward with a 0.7% parasitemia. This parasitemia subsequently developed to 64% and was accompanied by a 9% PCV, and a 1:160 CF titer.

An aliquot of the lysed material used to infect calf 500 was centrifuged 40 minutes, 5°C, at 18,000 RPM (over 35,000 X G) Splenectomized calf 206 was injected subcutaneously with 5 ml of the supernate, care being taken to avoid contamination from the sediment. Calf 206 did not show evidence of anaplasmosis, remaining negative for over 90 days.

Conclusions:

Infectious particles that were obviously present in the lysed cells were apparently thrown down, even though suspended in an isotonic solution. This is in contrast to infectious particles in plasma subjected to the same forces of gravity. This study leads us to hypothesize:

1.) that the particle size or density of the infectious organism found in the red cell is different than found in plasma .

2.) that plasma and the infectious particle have a specific gravity practically the same, and that plasma specific gravity is different than that of VB.

3.) that the infectious particles found in RBC have an affinity for RBC stroma, on which they congregate and are hence taken down with centrifugation.

Further studies of the physical nature of A. marginale are indicated and are being pursued as time permits.

Anaplasma Antigen Study Part I

Antigen Fractionation and Titrations

The development of serologic antigens for use in the diagnosis of anaplasmosis has been a highly successful phase of reasearch on this disease. As early as 1949, workers in the USDA described a complement-fixation antigen, which following numerous modifications, has become a standard serologic procedure for the diagnosis of anaplasmosis. In addition and some years later workers at Illinois, in cooperation with Diamond Laboratories, produced a capillary-tube agglutination antigen which some thought to be an improvement over the CF test. Most recently the USDA has reported a card agglutination test, which is a simple, rapid field test that can be done on the farm, producing results equally as reliable as the more complicated CF test. In addition to these diagnostic procedures, there are numerous accounts in the literature of various other antigen-antibody systems that work for the diagnosis of anaplasmosis, but which are less reliable or impractical in one way or another.

Our primary interest in *Anaplasma* antigens is not so much the development of additional diagnostic tests, as the location, isolation, and purification of antigens that may be useful in the development of a more satisfactory killed vaccine. The present killed vaccine (Anaplaz)* is considered a crude product in that many blood antigens are present which do not enhance the immunizing properties of that vaccine, and which actually

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* Fort Dodge Laboratories, Fort Dodge, Iowa

create the problem of producing blood type antibodies which are very likely responsible for hemolytic anemias seen in recent years in some calves born to cows having been vaccinated with these antigens. It is probable that a completely pure antigen, while desirable, may not be necessary to eliminate the problem of blood type antibody production in dangerous levels. If we could produce a partially purified antigen, eliminating 90% or more of the stroma, it is probable that a more effective killed vaccine could be produced.

For these reasons antigens have been prepared, fractionated, and tested for antigenicity using the CF test as a model for measuring this activity.

Materials and Methods:

Antigens have been prepared from 3 splenectomized calves showing acute anaplasmosis. Calf 229 had a 22% PCV and a 47% parasitemia; calf 500 had a 17% PCV and 64% parasitemia; Calf 142 had a 17% PCV and a 45% attenuated *A. marginale* (Diamond Laboratories) parasitemia at the time blood was harvested for antigen production on day 1, and on day 2 showed a 16% PCV and a 47% parasitemia, at which time a second antigen was made. A total of 650 ml blood was taken from 229, 600 ml from 500, and 320 ml from 142 on day 1, and 320 ml on day 2. Six antigens were prepared from 229 and are designated· 229-1, 229-2, 229-3, 229-4, 229-5 and 229-6. Four antigens were prepared from 500 and were labeled 500-1, 2, 3, and 4. Eight antigens were prepared from 142 on day 1 and were labeled 142-1-2-3, etc. Five antigens were prepared from 142 on day 2 and were labeled 142A-1, 142A-2, etc.

All antigens were prepared from packed RBC that had been washed 4 times at 2000 RPM - 10 minutes in veronal buffer (VB). Plasma antigens were prepared from cell free plasma, separated from blood on the same bleeding dates as used for red cell antigens.

The antigen titrations were conducted on increasing amounts of an arbitrary dilution with the end point being the amount of antigen giving 4+ or complete fixation of 2 units of complement in the presence of Anaplasma positive bovine serum having a titer of 1:40 A hemolytic system was standardized with 2% sheep cells and 2 units of amboceptor. All antigens were checked for anti-complementary activity using similar antigen amounts with negative or normal bovine serum.

A sample protocol is given below for antigen 229-2.

Tube	1	2	3	4	5	6	7	8	9
Antigen Amount:	0.05	0.10	.15	.20	.25	.30	.40	.50	.60
Positive Serum:		1	2	3	4	4	4	4	4
Negative Serum:	-	-	-	-	-	-	-	-	-

A 1:1 antigen dilution was used.

1U = 0.25 ml of the undiluted antigen 229-2

In this instance 67 ml of a previous total of 282.6 ml, 229-1, was used and then concentrated into 21.6212 ml, consisting of 20.0 ml VB and 1.6212 gms of sediment.

Total antigenic units were calculated as follows:

0.25 : 1U = 21.6212 : X

.25X = 21.6212 = ; X = 86.48 units present in 21.6212 gms.

Since 67 represents only 23.7% of the total volume a comparable number of units = 86.48/.237 = 365 units for comparison to 229-1.

70 ml packed cells were used in the preparation of these antigens so 365/70 = 5.2 units/ml packed cells. As a measure of concentration the units per gram of sediment were calculated as 21.6212/1.6212 = 13.3 units/gm.

In some instances 4+ end points were not obtained with the initial dilution. If after dilution adjustments 4 or more points of partial fixation were observed, a regression analysis was conducted and a projected end point calculated. If fewer than 4 tubes of partial fixation were observed in undiluted antigen, titers were recorded as 0.

Flow charts have been prepared for each of the 4 major antigen groups and are listed as Figures 22, 23, 24, and 25.

Antigen 229-1:

1.) 70 ml packed RBC were lysed in 10 volumes distilled water.

2.) Lysed cells were held overnite at 5°C.

3.) Lysed cells were centrifuged 40 minutes at 35,000 x G. The supernate was decanted and saved. The sediment was collected and resuspended in equal volume of distilled water, using the Vir-tis at low speed.

4.) This suspension was again centrifuged 40 minutes at 35,000 x G in a refrigerated centrifuge.

5.) The supernatant liquid was discarded. The sediment remaining showed a marked division, a light weight sediment which could be poured off and a packed, more dense, sediment.

6.) The light weight sediment was poured off. The weight of this material was 56.6 grams. This was mixed full speed in a Vir-tis with 226 ml VB to give a 20% suspension, labeled and frozen at -20°C until titrated.

Antigen 229-2

This antigen was an attempt to concentrate antigen 229-1. The 1st 6 steps were identical.

1.) A total of 67 ml antigen 229-1 was thawed and centrifuged 40 minutes at 35,000 x G under refrigeration.

2.) The sediment was saved and resuspended in VB with light sonication and once again centrifuged 40 minutes at 35,000 x G.

3.) The supernate was discarded. The sediment was resuspended in VB with light sonication (just enough to eliminate visible particles) and once again centrifuged 40 minutes at 35,000 x G.

4.) The sediment was collected, weighed, and re-suspended in 20 ml VB. Sediment weight was 1.6212 gms. This material was sonicated 1 minute at 80 watts on the sonifier.

Antigen 229-3

This antigen consisted of the supernate collected following step 1 in the preparation of Antigen 229-2.

Antigen 229-4

This antigen was the packed or dense sediment observed in step 5 of Antigen 229-1.

1.) This dense sediment weighed 3.221 gms. This was mixed at full speed in a Vir-tis tissue homogenizer "45", with 61.2 ml VB to give a 5% suspension, which was labeled 229-4 and stored till use.

Antigen 500-1

1.) 56 ml washed, packed RBCs were lysed in 10 volumes distilled water.

2.) Lysed cells were held overnight at 5°C.

3.) Lysed cells were sonicated 3 minutes at 120 watts.

4.) Centrifuged 40 minutes at 35,000 x G., and the sediment collected.

5.) Sediment was washed with cold distilled water, sonicated sufficient to bring sediment into suspension, and centrifuged 40 minutes at 35,000 x G.

6.) The supernate was discarded. The sediment showed a dark button overlayed by a fluffy sediment.

7.) This fluffy sediment weighed 3.183 gms. A 5% suspension was prepared by adding 60.5 ml VB. This was ground in the Vir-tis, bottled, and frozen.

Antigen 500-2

This antigen was the dense sediment observed in Step 6.

1.) The dense sediment weighed 0.5446 gms. This was mixed with 54 ml VB, using a sonicator to suspend the sediment. Antigen was labeled, bottled, and frozen.

Antigen 500-3

1.) The supernate was collected following centrifugation of Antigen 500-1, Step 4.

2.) The pH of this supernate was lowered to 5.0 by the addition of 1 N HCl.

3.) After the pH had stabilized at 5.0 the material was left overnight at $5^{o}C$.

4.) The next day the pH 5.0 supernate was centrifuged 40 minutes at 35,000 x G.

5.) Supernate was discarded, the sediment weighed 3.3693 gms. The sediment was mixed by sonication with 81 ml VB to give a 4% suspension.

Antigen 500-4

1.) 56 ml washed, packed RBCs were lysed in 10 volumes distilled water saturated with CO_2.

2.) This preparation was allowed to stand overnight at $5^{o}C$.

3.) The floculating precipitate was collected by centrifugation, (40 minutes at 35,000 x G) the supernate being discarded.

4.) The sediment was washed 2 times in distilled water saturated with CO_2, with the sediment being thrown down each time by centrifugation 40 minutes at 35,000 x G.

5.) The final sediment weighed 3.183 gms. This was added to 60.5 ml VB to give a 5% suspension, and mixed in a Vir-tis grinder. It was then labeled and frozen.

Antigen 142-1 (light sediment)

1.) 54 ml washed, packed RBCs were lysed in 10 volumes of distilled water.

2.) After an overnight storage at $5^{o}C$, lysed cells were centrifuged 40 minutes at 35,000 x G. in a refrigerated centrifuge.

3.) The resulting sediment was washed in distilled water, mixing with the Vir-tis grinder at low speed.

4.) The material was centrifuged a second time 40 minutes at 35,000 x G.

5.) The fluffy sediment was poured off and weighed 25.3 gms. A 20% suspension in VB was prepared by adding 101.2 ml VB. The sediment was suspended by sonication, labeled and frozen before testing.

Antigen 142-2

1.) 42 ml of 142-1 was taken and sonicated a second time, then centrifuged 40 minutes at 35,000 x G.

2.) The sediment following centrifugation weighed .2344 gms. This was suspended in VB by sonication and labeled 142-2 prior to testing.

Antigen 142-3

1.) The supernate following Step 1 of Antigen 142-2 was saved and labeled 142-3.

Antigen 142-4 (Heavy sediment)

1.) Steps 1 thru 4, 142-1, were the same for this antigen.

2.) A dense, or heavy sediment remaining in the tube after the removal of 142-1 weighed 5.013 gms. To this sediment was added 95.2 ml VB and then sonicated. This material was then labeled, and frozen before testing.

Antigen 142-5

1.) 20 ml of 142-2 antigen was centrifuged 40 minutes at 35,000 x G.

2.) The sediment collected was re-suspended in 20 ml VB by sonication, labeled and frozen.

Antigen 142-6

1.) The supernate from 142-5 sediment was saved and tested for evidence of antigenicity.

Antigen 142-7

1.) 212 ml of plasma was collected when citrated blood was centrifuged for 20 minutes at 2,000 RPM.

2.) This plasma was centrifuged 2 times, 10 minutes each at 650 x G to remove all traces of cellular material.

3.) The plasma was diluted in 2 volumes of distilled water and allowed to stand overnight.

4.) Diluted plasma was centrifuged 1 hour at 35,000 x G.

5.) A small button of sediment was seen and saved, the supernate being discarded.

6.) This plasma sediment was suspended in 40 ml VB by sonifying 1 minute at 80 watts, labeled and frozen for testing.

Antigen 142-8 (plasma antigen)

1.) Antigen 142-7 after testing was centrifuged 1 hour at 35,000 x G.

2.) The sediment was suspended in 1/3 the original volume with VB.

Antigen 142A -1 (PCV = 16%, Parasitemia = 49%) 320 ml blood.

1.) 47 ml washed, packed RBCs were lysed in 10 volumes distilled water.

2.) The lysed cells, after standing in the refrigerator overnight, were centrifuged 40 minutes at 35,000 x G.

3.) The resulting sediment was suspended in 150 ml distilled water by sonication 1 minute at 90 watts, then centrifuged 40 minutes at 35,000 x G.

4.) The light sediment was poured off and washed with VB, centrifuged and re-suspended by sonication.

5.) This suspension was centrifuged 40 minutes at 35,000 x G.

6.) The resulting sediment was saved, weighed (0.623 gms) and suspended in 20 ml VB by sonication. This material was labeled and frozen for later titration.

Antigen 142A-2

1.) The heavy sediment following Step 3 of Antigen 142A-1 was collected and re-suspended in 35 ml VB by sonication.

2.) This suspension was centrifuged 40 minutes at 35,000 x G.

3.) The resulting sediment weighed 0.1403 grams which was suspended 27 ml in VB by sonication.

4.) This suspension was centrifuged 40 minutes at 35,000 x G.

5.) The resulting sediment weighed 0.1263 gms (90% of #3), was suspended in 15 ml VB by sonication, labeled and frozen prior to testing.

Antigen 142A-3

1.) The VB supernate collected after 142A-2, Step 2, was collected and labeled for testing.

Antigen 142A-4

1.) The VB supernate collected after 142A-2, Step 4, was collected and labeled for testing.

Antigen 142A-5 (plasma)

1.) Plasma was carefully removed with a syringe and needle after the initial centrifugation of citrated blood from Calf 142.
2.) This plasma was centrifuged 1 time for 10 minutes at 650 x G, then 1 time at 10,000 x G to remove all cellular components.
3.) The clear, cell free plasma was diluted with 4 volumes of distilled water and then centrifuged 1 hour at 35,000 x G.
4.) 16 ml VB was added to the resulting sediment, and was then suspended by sonication.
5.) This material was labeled and frozen prior to testing.

Results:

The total and relative activity of each antigen described is listed in Table 12. Antigens 229, 500, 142, and 142A showed both greater antigen yield and concentration in the dense or heavy sediment following water lysis and high speed centrifugation. The light sediment was, however, found to contain significant activity and is not made up entirely of non-antigenic material to be discarded. It can be greatly concentrated by rupturing cellular material by sonication followed by high speed centrifugation,

but in so doing a small amount of antigen is lost. It is believed, however, that the greater concentration more than offsets the amount that is lost. This treatment (sonication of light sediment) increased the antigenic concentration of 229-1 from 7 units/gram to 53.3 u/gm for almost an 8-fold increase, with a decrease of only 3.2% in the total antigen available. The light sediment in Antigen 142 showed an even greater increase in concentration following sonication, increasing from 16.7 u/gm of sediment in 142-1 to 582.2 u/gm of sediment in 142-2 for a 35-fold increase at an overall loss in antigenic material of only 2.6%

Attempts at further purification and concentrations of the heavy sediment in Antigen 142 by sonication and centrifugation showed an overall decrease in available antigen of 50%. The concentration in the final sediment was not determined due to our inability to obtain an accurate weight on such a small amount. The antigenicity concentration was probably greater, but the great loss in antigen mass suggests that a point of diminishing returns had been reached, and further wash cycles in VB were not indicated.

It is significant that all supernate antigens failed to show measurable activity. Antigens 229-5 and 500-3, in which precipitates occurred,following pH reduction to 5.0, and Antigens 229-3, 142-3, 142-6, 142A-3 and 142A-4 which were veronal buffer supernates following high speed centrifugation, were negative in every instance. It is possible that some activity was present, since the overall antigen quantities usually decreased slightly with each wash and centrifugation, but the quantities present, if they were present, were not measurable.

The other type antigen was the CO_2 lysed cells, which uniformly resulted in an antigenic mass roughly equivalent to the total light and heavy sediments observed in the water lysed antigens. In antigen 229 the total antigenic units of the water lysed sediments was 2095, whereas the CO_2 antigen showed 2013. In antigen 500 the total water lysed sediments showed 1940 units, whereas the CO_2 antigen showed 2547. These differences are just reversed, probably reflecting a slight error in titrations or loss in manipulation. The actual measured antigen amounts are remarkably similar.

This information, combined with the absence of measurable activity of supernate antigens even when concentrated by pH reduction, strongly suggests that the antigens measured are particulate antigens, which can and are thrown down by 35,000 x G for 40 minutes. There does not appear to be any marked denaturation of antigen by the CO_2 treatment, however, slight denaturation would not have been detected by these trials. The CO_2 antigen is less purified, and undoubtedly contains greater quantities of non-specific material as evidenced by the lower units per gram of sediment. The greatest antigen concentration was obtained in the heavy sediment following sonification. In 229, the CO_2 antigen had a concentration of 133 u/gm, in comparison to 537 for the heavy sediment 229-4 for a 4-fold difference. In 500, the CO_2 antigen had a concentration of 800 in comparison to 2003 for the heavy sediment 500-2 for a 2.5 fold difference.

Plasma antigens, while apparently showing some specific fixation, were anti-complementary and could not be measured by these techniques. Efforts to overcome this characteristic have, to date, been unsuccessful on bovine sera.

Antigens prepared from splenectomized Calf 142 infected with the Diamond attenuated *A. marginale* did not differ significantly from those prepared from the virulent organism.

Conclusions:

These studies strongly suggest the possibility of successful purification and concentration of immunizing antigens. A water lysed antigen is preferable, and it is possible that future efforts will include sonication of the light sediment and inclusion in a final vaccine product.

Figure 22

229 Antigens

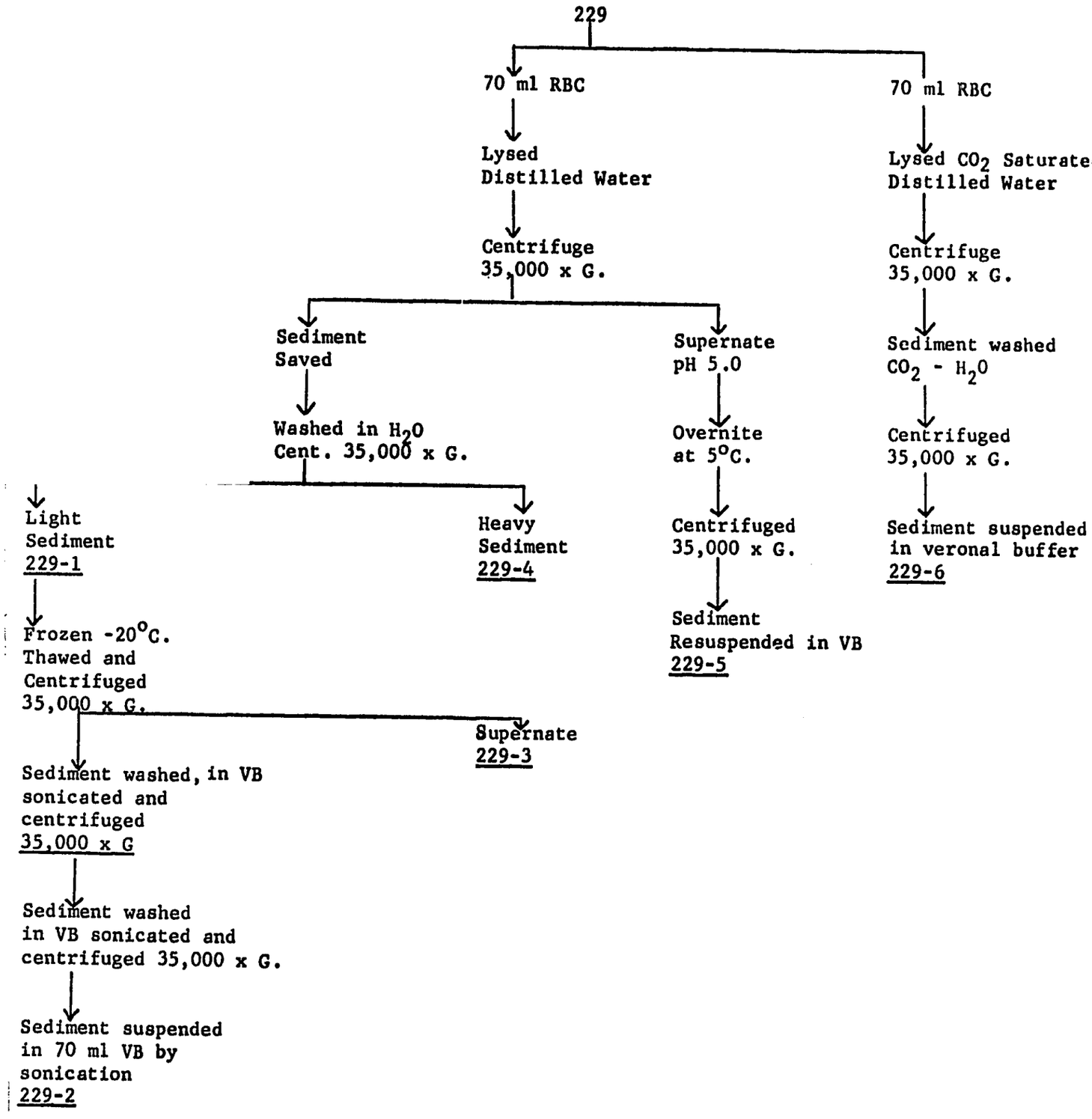


Figure 23

500 Antigen

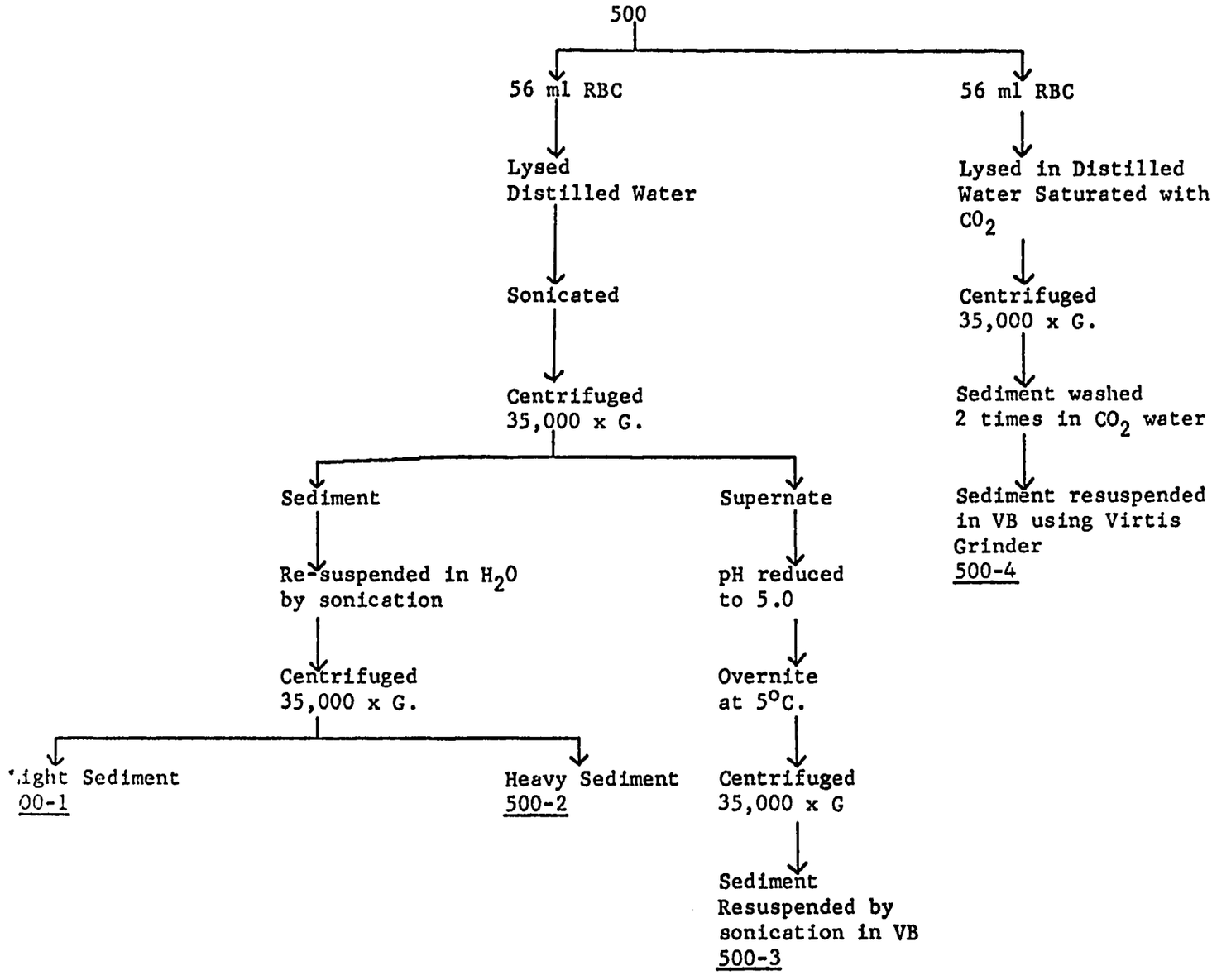


Figure 24

142 Antigen

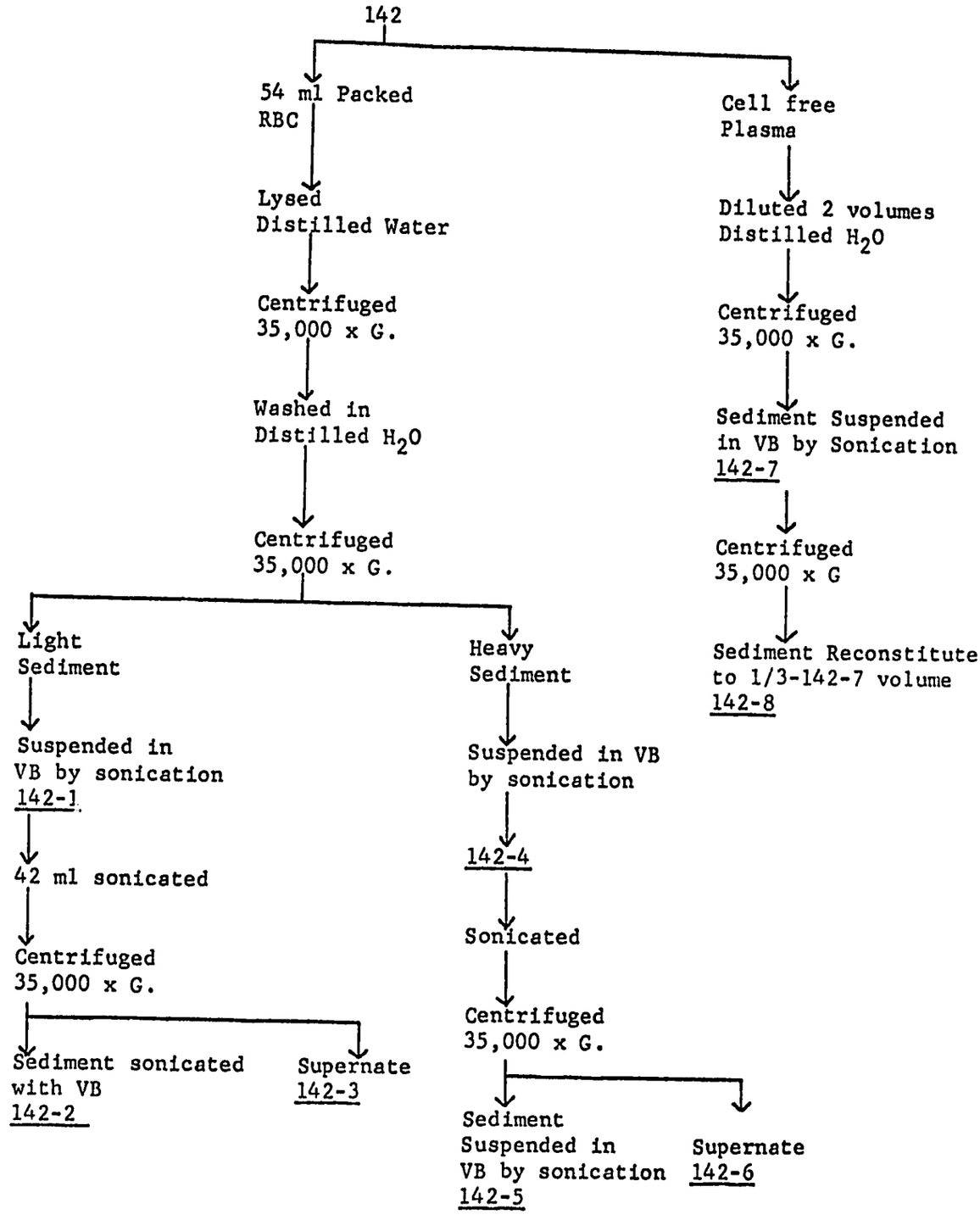


Figure 25

142A Antigen

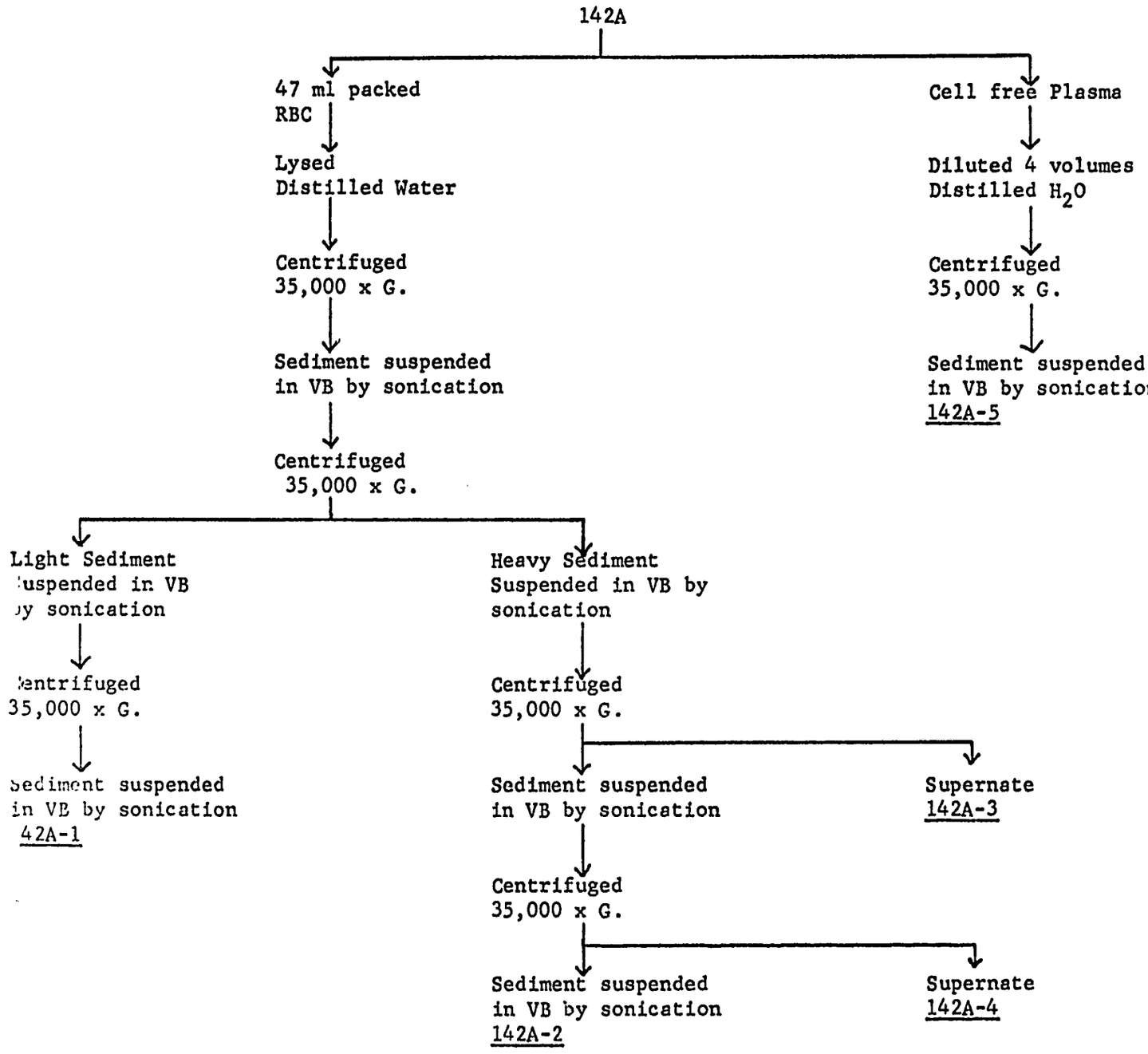


Table 12

Relative Antigenic Activity

Antigen	Type of Antigen	Total Antigenic Units Recovered	Units/ml Packed RBC	Units/ gm of Sediment
229-1	RBC	377	5.4	7
229-2	"	365	5.2	53.3
229-3	"	0	0	0
229-4	"	1718	24.5	537
229-5	"	0	0	0
229-6	"	2013	28.8	133
500-1	RBC	849	15.2	267
500-2	"	1091	19.5	2003
500-3	"	0	0	0
500-4	"	2547	45.5	800
142-1	RBC	422	7.8	16.7
142-2	"	411	7.6	582.2
142-3	"	0	0	0
142-4	"	2004	37.1	400.0
142-5	"	1002	18.5	NT
142-6	"	0	0	0
142-7	Plasma	AC	AC	AC
142-8	"	AC	AC	AC
142A-1	RBC	442	8.8	661
142A-2	"	378	8.04	2993
142A-3	"	0	0	0
142A-4	"	0	0	0
142A-5	Plasma	AC	AC	AC

AC: Anticomplementary

Anaplasma Antigen Study Part II

Reactive Characteristics of USDA Standard CF Antigen

The exact method of production for USDA antigen is not known even though basically it is known to be a mixture of water and CO_2 lysed antigen. In the past we have reported a marked increase in antigenic titer following sonication. Theileria antigens have been shown to increase in titer when solubilized with high pH buffers (G. Corry thesis, 1970).

In order to more fully understand the active antigen in USDA, and to further explore ways of increasing titer and possibly purification, a series of tests were set up using the USDA antigen as a basic unit, and altering the antigen by a series of physical modifications.

The following preparations were made and then titrated against positive serum (1:40 titer).

A regression analysis was conducted on each titration to better establish the 4+ endpoint. This method enabled us to take into account the incomplete reaction, and the progression for each antigen. All CF readings were conducted by visual examination and estimation of the degree of fixation on a 1-40 scale.

Series 1

USDA #1: This antigen was a control. No treatment other than thawing and manually shaking. It was diluted 1+4 (1:5) and titrated.

USDA #2 USDA #1 antigen was sonicated 2 minutes at 80 watts and titrated 1+4 (1:5) dilution.

USDA #3: USDA #1 antigen was diluted 1+4 (1:5), sonicated 2 minutes at 80 watts, then titrated.

USDA #4: USDA #3 antigen was centrifuged 30 minutes at 20,000 x G, and the supernate tested for activity.

USDA #5: USDA #1 was diluted 1+4 (1;5) in VB pH 8.5. The pH was brought up to 10.0 by adding 0.1 N NaOH. This material was then sonicated 2 minutes at 80 watts. The antigen was titrated at this point.

USDA #6. USDA #5 was centrifuged 30 minutes at 20,000 x G, and the supernate checked for activity.

Series #2

2-USDA #1: Control - same as USDA #1 in Series 1.

2-USDA #2: Same as USDA #2 in Series 1.

2-USDA #3: Antigen 2-USDA #2 after sonication was centrifuged 30 minutes at 35,000 x G. The sediment was suspended in VB pH 7.2 to the original volume by sonication. The antigen was titrated in a 1+5 (1:6) dilution.

2-USDA #4: The supernate from 2-USDA #3 was tested undiluted for antigenic activity.

2-USDA #5: Antigen 2-USDA #1 was centrifuged 30 minutes at 35,000 x G. The sediment was suspended in VB pH 7.2 to the original volume by sonication, diluted 1+5 (1:6) and titrated.

2-USDA #6: The supernate from 2-USDA #5 was tested undiluted for antigenic activity.

Results:

Antigen units per ml are presented in Table 13 for Series 1 and 2 antigens. In Series 1, as expected, sonication was responsible for a greater than 2 fold increase in CF titer. This increase occurred irrespective of when sonication was applied. An increase in pH from 7.2 to 10.0 did not influence antigen titer. Centrifugation 30 minutes at 20,000 x G was sufficient to remove all measurable antigens in USDA #4, however, in USDA #6 when the pH was increased to 10, there was a measurable antigen response. This is the first time an apparent soluble antigen has been found active in the CF test. This trial needs to be repeated using greater centrifugal forces before the assumption can be made that a soluble CF antigen for anaplasmosis does occur under these circumstances.

The results of Series 2 are similar in that the control antigen was very nearly the same titer. A similar but not so marked an increase occurs with sonication. A wash of sonicated and unsonicated antigen resulted in a slight loss of titer, but no evidence of antigen presence was detected in supernates.

Conclusions:

The solubilization of *Theileria* antigens at pH 10, reported by Corry, does not apply to *Anaplasma* antigen, at least those treated with CO_2 distilled water. There was some slight evidence that antigens remained in the supernate following high speed centrifugation when treated with pH 10 buffers, which did not occur with pH 7.2 buffers. Washing antigen sediments is accompanied by a slight loss in potency, which cannot be accounted for. The drop, in some instances, is so small that mechanical factors may be involved with no actual loss occurring in the supernate.

Table 13

Modifications of USDA Antigens

Antigen	Type	Antigen Units/ml.
Series 1		
USDA #1	Control	20.4
USDA #2	Sonicated	52.6
USDA #3	dil 1:5 - sonicated	52.6
USDA #4	Supernate from #3	0
USDA #5	pH 10.0 - Sonicated	52.6
USDA #6	pH 10.0 - Supernate	1.7
Series 2		
USDA #1	Control	20.8
USDA #2	Sonicated	43.5
USDA #3	1 wash of #2 in VB - Sonicated	40.0
USDA #4	Supernate from #3	0
USDA #5	1 wash of #1 in VB - Sonicated	32.3
USDA #6	Supernate from #5	0

Anaplasma Antigen Study Part III

Small Volume Antigen

Previous work on the preparation of CF antigens has been done primarily with the idea of preparing diagnostic aids and has generally been approached from the point of view of using maximum volumes of blood showing maximum parasitemias. Situations are now developing, in attempts to cultivate A. marginale, where it would be desirable to detect antigens occurring in low concentrations. The fluorescent antibody techniques are suited for this, however, there are drawbacks in that it is primarily a qualitative, not quantitative test, and also it is frequently impractical when non-specific fluorescence becomes a problem.

It is also known that in some instances initial or elementary bodies occur in the development of anaplasmosis that are not visible with the light microscope, but which are probably antigenic. A serologic technique to accurately measure antigenic titers in animals developing and recovering from anaplasmosis would provide a valuable parameter for better evaluating the infectious process, and the influence of external influences on the infection.

A few preliminary trials have shown detectable levels of antigenic activity in blood showing relatively low level A. marginale parasitemia. In these trials a standardized antigen protocol has been developed using relatively small volumes of blood for the preparation of CF antigen to better determine the practicality of this approach as an indicator of Anaplasma growth.

Materials and Methods:

The following standard antigen protocol has been adopted and basically followed in the preparation of 17 antigens from 11 calves showing ascending or descending low level or negative parasitemias.

1.) Based on PCV, sufficient blood is collected in citrate to give 8 ml packed, washed red blood cells (RBC), after 4 washings. This is usually about 40-50 ml whole blood.

2.) RBCs are washed 4 times by centrifugation, 10 minutes at 650 x G, in VB, removing as nearly as possible the buffy coat.

3.) 8 ml washed RBC are then lysed in 4 volumes, or 32 ml distilled water, and allowed to stand at least 3 hours, and sometimes overnight.

4.) Lysed cells are centrifuged 35,000 x G for 40 minutes. Hereafter every centrifugation is at this speed and time.

5.) Sediment suspended in distilled water by sonication and centrifuged.

6.) Sediment suspended in distilled water by sonication and centrifuged.

7.) Sediment suspended in VB by sonication and centrifuged.

8.) If color is gone at this step the sediment is suspended by sonication using either 4 or 10 ml VB, labeled, frozen and later titrated.

8a.) If color is not gone, a final VB wash is given with sediment then being suspended and titrated.

Antigen titers are determined by using the undiluted or diluted antigen in progressively increasing amounts shown:

0.05 0.10 0.15 0.20 0.25 0.30 0.04 0.05 0.06

The dilution used is arbitrarily determined, and if the antigen activity does not fall within our range, a second dilution is made, unless no activity was recorded in the undiluted material. In most instances undiluted antigens were used.

All tests were conducted using 2 units guinea pig complement, 2 amboceptor units added to 2% sheep cells, and a single Anaplasma positive serum with a 1:40 titer along with a control negative serum for the detection of AC activity.

Since the reconstituting volume was different in some instances, results are expressed as total number of units present in 8 ml packed RBC. The antigen end point, or dilution at which 4+ fixation occurred, was determined by a regression analysis, taking into consideration the incomplete reactions prior to the first 4 + reaction. This technique was used to project an end point for antigens where partial fixation was encountered.

The antigen end point thus obtained was taken as 1 unit of antigen. Since all antigens were prepared from 8 ml packed RBC, with a final volume of 4 or 10 ml, an antigen value was calculated to give the total number of units in each antigen. For example:

If Antigen A had a 4+ end point of 0.30 ml of a 1:2 dilution, and the sediment obtained from 8 ml packed RBC had been suspended in 10 ml VB, then:

1 U = .30 of a 1:2 or 0.15 of a 1:1

10/0.15 = 66.7 units

All antigens were similarly recorded for a standardized comparison.

A comparison of antigen titers was made to parasitemia and to the stage of infection whether the parasitemia was ascending or descending in nature.

Results:

Of 17 antigens prepared from blood showing 0 to 12% A. marginale parasitemias, 14 showed detectable amounts of antigenic activity. CF antigens were not detected in two calves with a 0 parasitemia and 1 calf with a 0.3% descending parasitemia. Antigens were detected in calf blood showing a 0.2% ascending parasitemia and in all others above 0.7% (Table 14).

A linear regression analysis on those antigens showing activity was conducted to determine if a correlation exists between parasitemia and antigenic units. These results are presented in Figure 25. A highly significant positive correlation occurs between parasitemia and antigenic unit (r = 0.823), with antigenic units increasing (regression coefficient = 3.84) in association with higher parasitemias.

To further characterize this association, a similar analysis was conducted on 6 antigens from animals having a descending parasitemia and on 8 antigens from animals having an ascending parasitemia. The regression lines are plotted on Figure 25 and show some marked differences. Antigens from animals showing the ascending parasitemia showed a significant

relationship between parasitemia and anemia at a probability of error of <0.01, whereas this relationship only reached $P<0.05$ for the descending parasitemia. The slopes are quite different.

Even though a linear relationship exists between parasitemia and antigenicity, there does appear another factor of relative activity for a given parasitemia. The number of antigenic units per each unit of parasitemia (each 1%) was therefore determined and then compared to the actual parasitemia in calves with ascending and descending parasitemias. The relative antigenicity in ascending infection was higher in those animals with a low parasitemia and decreased proportionately with an increase in actual parasitemia. This relationship was significant at the 0.05 level.(Figure 26). Animals showing a descending parasitemia failed to show a significantly different relative activity with the lower parasitemias.

Conclusion and Discussion:

1.) A direct correlation exists between antigenic units and parasitemia with a t value of 5.81 ($P<0.01$).

2.) In ascending infections this correlation is highly significant with a t value of 9.93 ($P<0.01$).

3.) In descending infections the correlation, while significant, has a t value of 2.90 which is significant at the 0.05 level.

4.) A marked difference in the regression line was noted when descending and ascending infections were compared.

5.) Relative to parasitemia, the number of antigenic units appears to decrease more rapidly in the descending infections. It is hypothesized that invading initial bodies, or immature sub-

microscopic bodies are not present in a descending infection to the same extent as seen in ascending infections, hence, low parasitemias of descending infections are accompanied by a lower antigenic concentration.

6.) It is further assumed that in descending infections the maturation process of existing Anaplasma bodies would in turn increase the quantity of antigen for each parasitized cell , thus accentuating the steepness of the slope, particularly in parasitemias measured soon after the peak of infection.

7.) The analysis of a ratio, or relative change in antigenic activity per unit change in parasitemia, showed a relationship, as noted in Figure 26, with greater changes occurring in antigenic units in the course of infection. In other words, the antigenic units per 1% parasitemia was greater in very early ascending infections. This observation was statistically significant at the 0.05 level. This same relationship was not demonstrable in descending parasitism. The hypothesis is indicated that the sub-microscopic, or initial bodies, are more numerous in early ascending infection, that they are antigenically active, and that the CF test is capable of detecting these antigen units.

Successful CF antigens can be prepared from relatively small volumes with low parasitemias. Future studies will include frequent antigen preparations during the course of induced infection to better characterize the findings noted in these studies.

Table 14

Detection of CF Antigens in Low Level Parasitemias

Animal	Date	PCV	Parasitemia	Total Antigen Units in 8 ml. Packed RBCs
272	12-11-70	11	4	15.4
219	12-14-70	14	5	28.6
222	12-14-70	18	0	0
226	12-14-70	19	0.3	0
245	12-14-70	16	7.0	29.4
248	12-14-70	19	0	0
264	12-14-70	17	0.8	16.0
245	2-2-71	24	0.2	9.3
245	2-5-71	27	1.5	15.6
245	2-8-71	27	5.0	25.0
217	2-10-71	15	8.0	38.6
276	2-19-71	25	12.0	43.5
276	2-22-71	18	5.5	55.6
142	3-1-71	13	0.7	9.1
217	3-4-71	20	2.0	9.6
276	3-4-71	20	2.0	12.7
405	3-9-71	12	9.0	55.9

Figure 25

Linear Regression Lines Indicating the Correlation of Antigenicity and Parasitemia

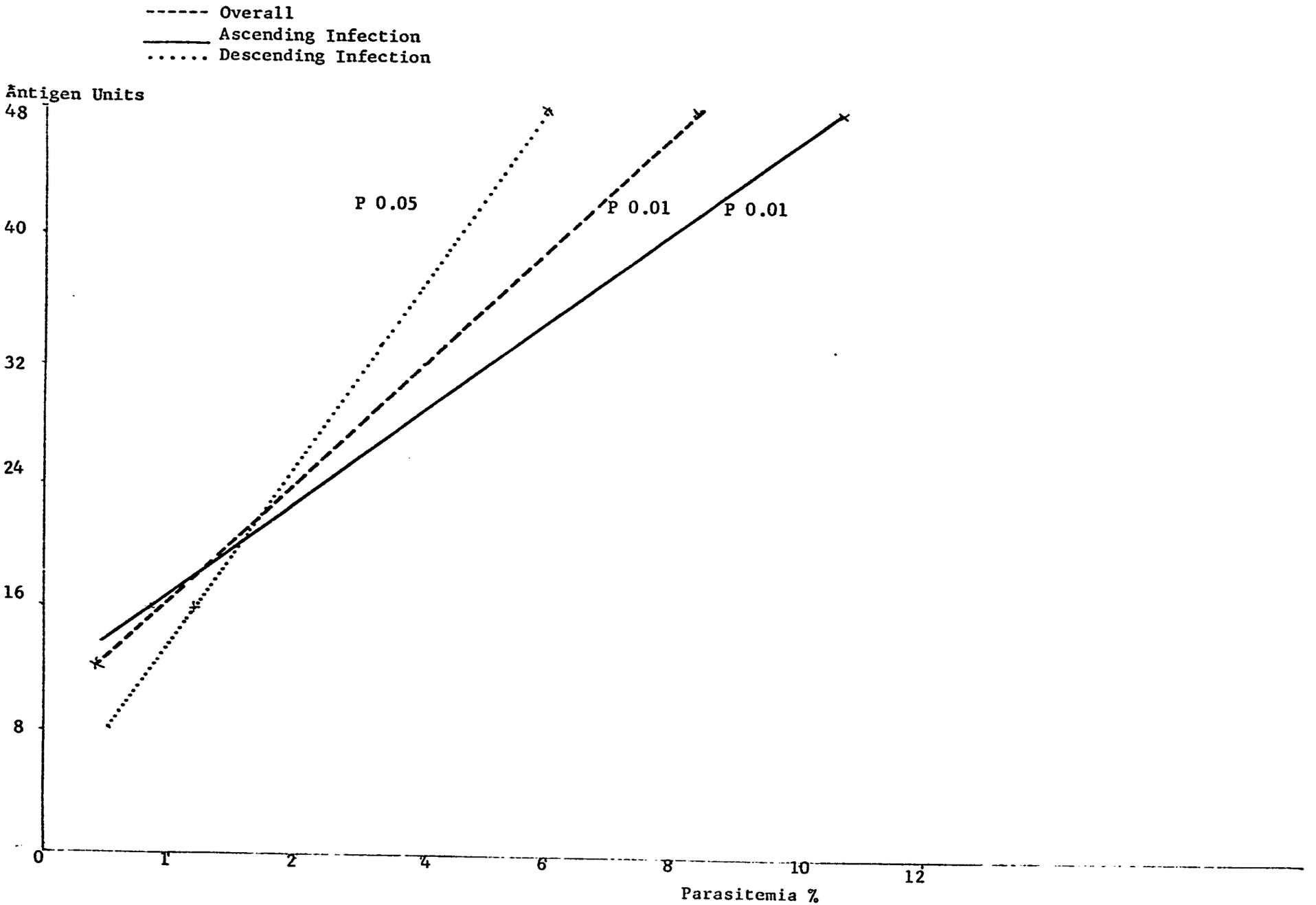
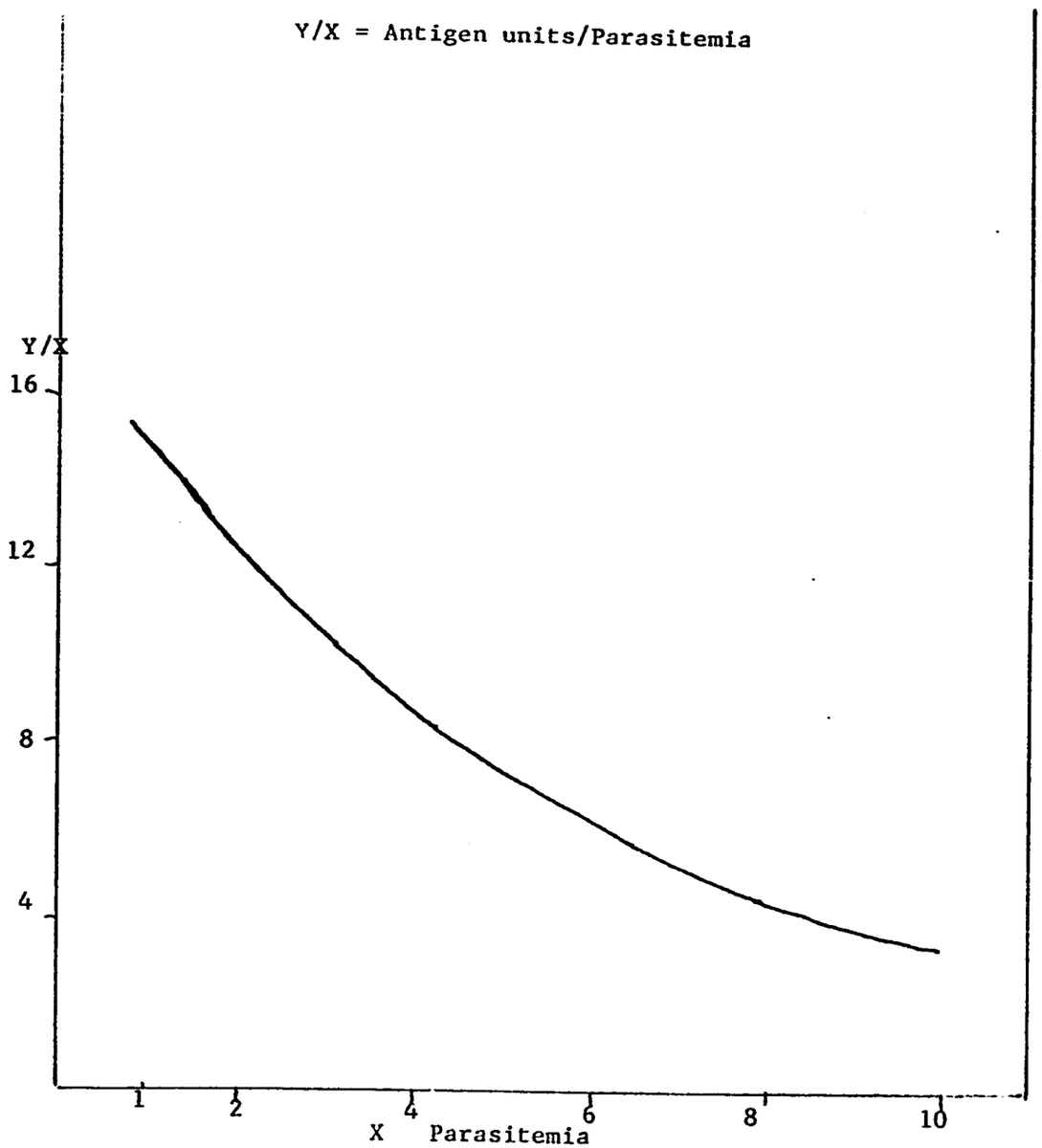


Figure 26

Relative Antigenicity
(P<0.05)

In an Ascending Infection



Anaplasma Antigen Study Part IV

Sheep Cells as a Source of *A. marginale* Antigen

It is reasonable to assume that *Anaplasma* CF antigens can be prepared from sheep blood, but efforts along these lines have not been reported. Sheep are susceptible to *Anaplasma marginale*, but usually develop extremely low level parasitemiaseven in splenectomized animals. The *A. marginale* attenuated strain (Diamond Laboratories), on the other hand, is responsible for higher parasitemias, which are accompanied by clinical evidence of infection. For this reason a sheep infected with this stain was selected. Studies reported in Part III further illustrate that extremely high parasitemias are not essential, and that measurable antigens are detected when the initial volume of RBCs is reasonably low. With this background, this experiment was approached with some degree of confidence that a successful antigen was possible.

Splenectomized sheep, 285, was exposed to *A. marginale* (attenuated), by injecting it I.V. with 40 ml packed RBCs showing a 5% *A. marginale* parasitemia. A total of 320 ml blood was collected in citrate 9 days later when the PCV in 285 was 17% and the parasitemia was 12%. This was a peak parasitemia. After the collection of blood for antigen the parasitemia rapidly decreased.

Two plasma and one RBC sediment antigen were prepared in steps as listed.

Plasma Antigens

1.) Plasma was carefully removed from packed RBCs after centrifugation 20 minutes at 650 x G.

2.) The plasma, even though visibly cell free, was centrifuged a second time 30 minutes at 10,000 x G to remove all cellular debris.

3.) The cell free plasma was then diluted with 4 volumes of distilled water, approximately 220 ml plasma being diluted with 880 ml water.

4.) This material was centrifuged 1 hour at 35,000 x G until sediment was collected from the entire 1100 ml in 8 centrifuge tubes. The supernate was discarded.

5.) A sediment weight of 0.4431 grams was harvested and then suspended in 38 ml distilled water by sonication.

6.) This material was centrifuged 1 hour at 35,000 x G.

7.) The sediment, weighing 0.0833 grams, was suspended in 15 ml VB and titrated for antigenic activity.

PA-2 1.) A second plasma antigen was prepared from the supernate obtained following step 6 in the previous (PA-1) antigen.

2.) This supernate (38 ml) was made isotonic by adding .34 gms NaCl and was then tested for antigenic activity.

RBC Sediment Antigen

1.) RBCs were washed 4 times in VB removing the buffy coat as much as possible without sacrificing RBC. The cells were fragile with some evidence of hemolysis even after the last wash.

2.) Approximately 40 ml packed RBCs were lysed in 10 volumes of distilled water.

3.) After 3 hours at 5°C the lysed cells were centrifuged 40 minutes at 35,000 x G.

4.) The sediment was re-suspended in distilled water by sonication and again centrifuged 40 minutes at 35,000 x G.

5.) The sediment was again re-suspended in distilled water by sonication and again centrifuged 40 minutes at 35,000 x G.

6.) The sediment was suspended in VB by sonication and centrifuged 40 minutes at 35,000 x G.

7.) The resulting sediment weighed 2.5196 gms.

8.) This sediment was suspended in 50 ml VB and tested for antigenic activity.

Antigen titrations were conducted essentially as previously described in Part I of this section. The same method has been used to standardize antigenic activity, one based on the total antigen units recovered, second, the number of units per ml of the beginning material, (in this case either packed RBCs or plasma), and a third on a measure of concentration as expressed in the number of units per gram of final sediment.

Results and Discussion:

Antigen activity was detected in one plasma antigen and in the RBC sediment antigen. The relative activity of these antigens is tabulated in Table 15. The RBC sediment antigen was easily detected and showed a level of activity similar to bovine cell antigens, when the parasitemia is taken into consideration. The actual units per ml of packed RBC, 6.25, compares favorably to antigens such as 229-4 and 500-2, when allowance is made for the parasitemia.

The presence of specific, complement-fixing antigens of a particulate nature in plasma has not to my knowledge been previously reported. This study suggests that the infectious unit for anaplasmosis does occur outside the erythrocyte in sufficient quantity to measure serologically. Comparisons between the total antigenic units recovered from 320 ml of blood from sheep 285 shows 89% present in RBC and 11% present in plasma. The final sediment in the plasma antigen, weighing only 0.0833 gms, represents a concentration of antigenic units almost 4 times greater than the RBC sediments. There is no doubt that bovine red cell antigens would be entirely absent in this sheep plasma antigen. It is equally obvious, however, that unless this antigen could be accumulated in greater quantity, that it would have little or no value as an immunizing antigen.

Antigen titrations during the course of infection in sheep might reveal a period of maximum antigen concentration in plasma that would lend itself more readily to antigen concentraion. The elimination of anti-complementary activities by 1 wash with distilled water suggests that a similar approach could be made in bovine plasma. The extreme difficulty encountered in throwing down the infectious unit in undiluted plasma, even at 35,000 x G for 1 hour, also suggests that greater antigen concentrations might be a possibility if higher forces of gravity could be applied.

Table 15

Relative Activity of Sheep Origin Antigens

	Type Antigen	Total Antigenic units	Unit/ml of tissue used	Units/gm of sediment
PA-1	Plasma	32	0.145	383
PA-2	Plasma	AC	AC	AC
RBC - Sediment	RBC	250	6.25	99.2

Babesia Transmission by *Boophilus annulatus*
Neuvo Laredo

Work at Neuvo Laredo was concluded in December, 1970, about 18 months after it had been initiated. The limited objectives invisioned for this project were for the most part accomplished, but, in addition, a number of other interesting items were uncovered.

This work was referred to in the 1970 Annual Report, and that aspect dealing with efforts to transmit anaplasmosis to white-tailed deer by *B. annulatus* was fairly well completed at that time and will not be included in this report, but some additional information is available regarding *Babesia* transmission.

A total of 3 deer have been used in efforts to infect one or more with cattle *Babesia* by tick feeding or the inoculation of infected blood.

Deer 471:

This intact deer was infested on day 0 with 1 gram of *B. annulatus* larvae thought to be carrying *Babesia*. On the same day splenectomized calf 187 was similarly infested with 1 gram of larvae from the same source. Blood samples of 10 ml each were taken from deer 471; 7, 14, 21, 28, and 35 days after tick exposure and injected subcutaneously into splenectomized calf 182 to detect evidence of *Babesia* in deer 471. Engorged female ticks were harvested from deer 471, allowed to oviposit, after which larvae were collected and fed on splenectomized calf 182.

One gram of B. annulatus larvae failed to produce clinical evidence of babesiosis in deer 471. Calf 187, infested at the same time with larvae of common origin, developed babesiosis. On day 5, calf 187 showed a temperature of 104.0°F. On day 14, Babesia parasites were observed on thin blood smear. On day 16, a PCV of 14 was seen, and death from acute babesiosis occurred on day 17. The Babesia was identified (Dr. R.A. Todorovic) as B. argentina.

Splenectomized calf 182 inoculated on days 7, 14, 21, 28, and 35 with blood from deer 471 failed to show any evidence of babesiosis. This same calf, 70 days after the last inoculation of deer blood from 471, was infested with larvae collected from engorged female ticks having fed on D-471. Calf 182 did not develop signs of babesiosis following this infestation. Calf 182, 91 days after tick infestation, was challenged by inoculating 10 ml whole blood, subcutaneously, from a Babesia carrier calf. A 48% Babesia parasitemia was detected 16 days after injection, and death due to acute babesiosis occurred the following day.

Deer 475:

This intact deer was inoculated subcutaneously (S.C.) with 10 ml whole blood from a calf thought to be carrying Babesia on day 0. On the same day a similar inoculum was given S.C. to calf 177. Calf 183 was inoculated S.C. with 10 ml. whole blood collected from deer 475, 7, 14, 21, 28, and 35 days after the initial exposure. On day 56, deer 475 was splenectomized in an effort to induce a recrudescence, if an infection had actually been induced but not detected. Four weeks after splenectomy deer 475 was once

again inoculated S.C. with 10 ml whole blood from a Babesia carrier calf. Two weeks prior to this inoculation, calf 179 was given a similar exposure with blood from the same carrier calf and served as a control to demonstrate the carrier status of the donor calf. Calf 178 was inoculated S.C. with 10 ml. whole blood collected from deer 475; 7, 14, 21 and 28 days after the second exposure to infected bovine blood.

Intact deer 475 failed to show clinical evidence of babesiosis following the inoculation of 10 ml. Babesia infected blood. Splenectomized calf 177 had a 2% Babesia parasitemia 7 days after inoculation. The PCV dropped to 6%, 9 days after exposure, and the animal died shortly afterward on the 9th day. Attempts to pick up an infecting parasitemia by inoculating blood from D-475 into calf 183 was unsuccessful. Calf 183 showed no signs of babesiosis following exposure to deer 475 blood. To prove that calf 183 was susceptible, it was challenged with Babesia carrier blood 210 days after the last inoculation and was found to be fully susceptible to Babesia, death occurring 10 days after exposure.

Splenectomy of deer 475 was not followed by evidence of a relapsing infection.

Deer 475, now splenectomized, was once again exposed to babesiosis by the inoculation of blood from a carrier animal and again failed to develop evidence of infection. Calf 179, receiving blood from the same carrier animal used to inoculate D-475, developed evidence of acute babesiosis, with a Babesia parasitemia occurring 7 days after exposure and death occurring 5 days later. The repeated inoculation of splenectomized calf 178 with blood from D-475 failed to produce evidence of babesiosis. As a check for susceptibility calf 178 was challenged with 10 ml. whole blood from a Babesia carrier. Calf 178 proved to be fully susceptible with death occurring 14 days later.

Deer 124:

Intact deer 124 was infested on day 0 with 2 grams of B. annulatus larvae from a pool of larvae thought to be infected with Babesia. At the same time splenectomized calf 237 was infested with 2 gms. of larvae from the same source. After the release of B. annulatus larvae on deer 124, blood samples were taken weekly to determine if evidence of infection could be detected. Calf 238 was given 10 ml. S.C. whole blood from D-124, 7, 14, 21, 28, 35, and 42 days after larvae were released. A large number of engorged B. annulatus female ticks were recovered from 124, and were allowed to oviposit, with larvae being collected and pooled. When these larvae were ready to feed they were put on calf 240.

Results:

Deer 124 failed to show any evidence of Babesia infection after the introduction of infected ticks. Calf 237 showed a Babesia parasitemia 12 days after larvae release, and died 1 day later. Calf 238 failed to show evidence of babesiosis after repeated inoculations of blood from D-124. A Babesia challenge of calf 238 with 10 ml. carrier blood, given 28 days after the last deer blood inoculation, resulted in acute babesiosis. Splenectomized calf 240, receiving 2 grams of larvae from the engorged females taken from deer 124, failed to produce any signs of babesiosis.

Discussion:

Efforts to induce Babesia infection in white-tailed deer have been consistently negative in 3 deer. Exposure of deer 471 and 124 to B. annulatus larvae, shown to be capable of transmitting babesiosis to calves, has in both instances failed to produce evidence of infection. The possibility that Babesia

infected larvae might retain the infection during 1 life cycle on the deer was checked in both experiments. In each instance, the completion of 1 life cycle on the deer was sufficient to make the second generation incapable of transmitting Babesia infection to splenectomized calves. The possibility does exist that a transient, low level parasitemia in deer might occur. Attempts to detect this was made by bleeding each deer at weekly intervals and injecting this blood into susceptible, splenectomized calves. This method failed to recover Babesia from deer blood. It is possible, however, that a shorter bleeding interval may be needed, but this was impossible under the circumstances.

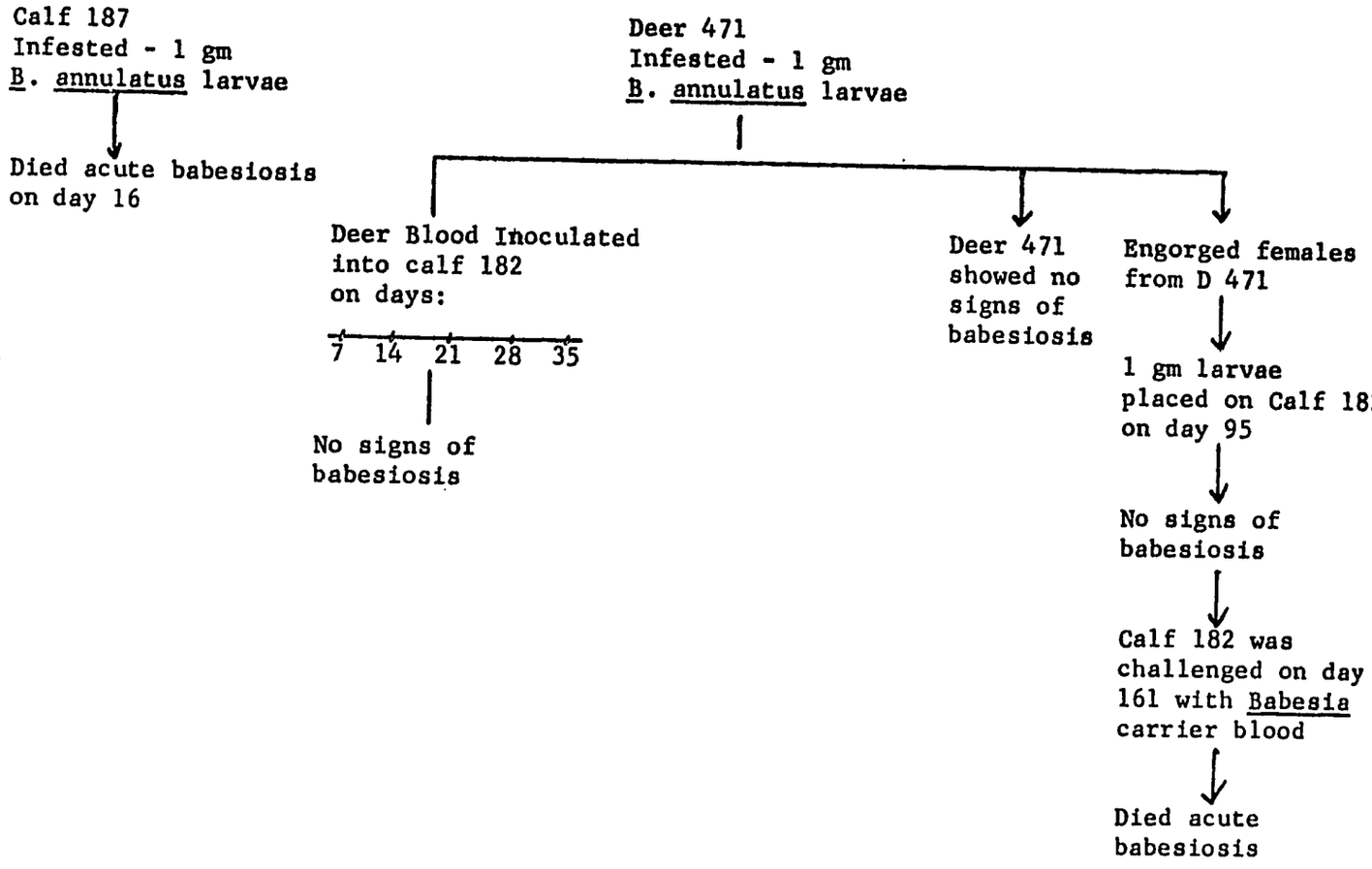
Efforts to infect deer by needle inoculation were equally unsuccessful. One week after the inoculation of proven infected blood, Babesia could not be recovered in splenectomized calves.

Conclusion:

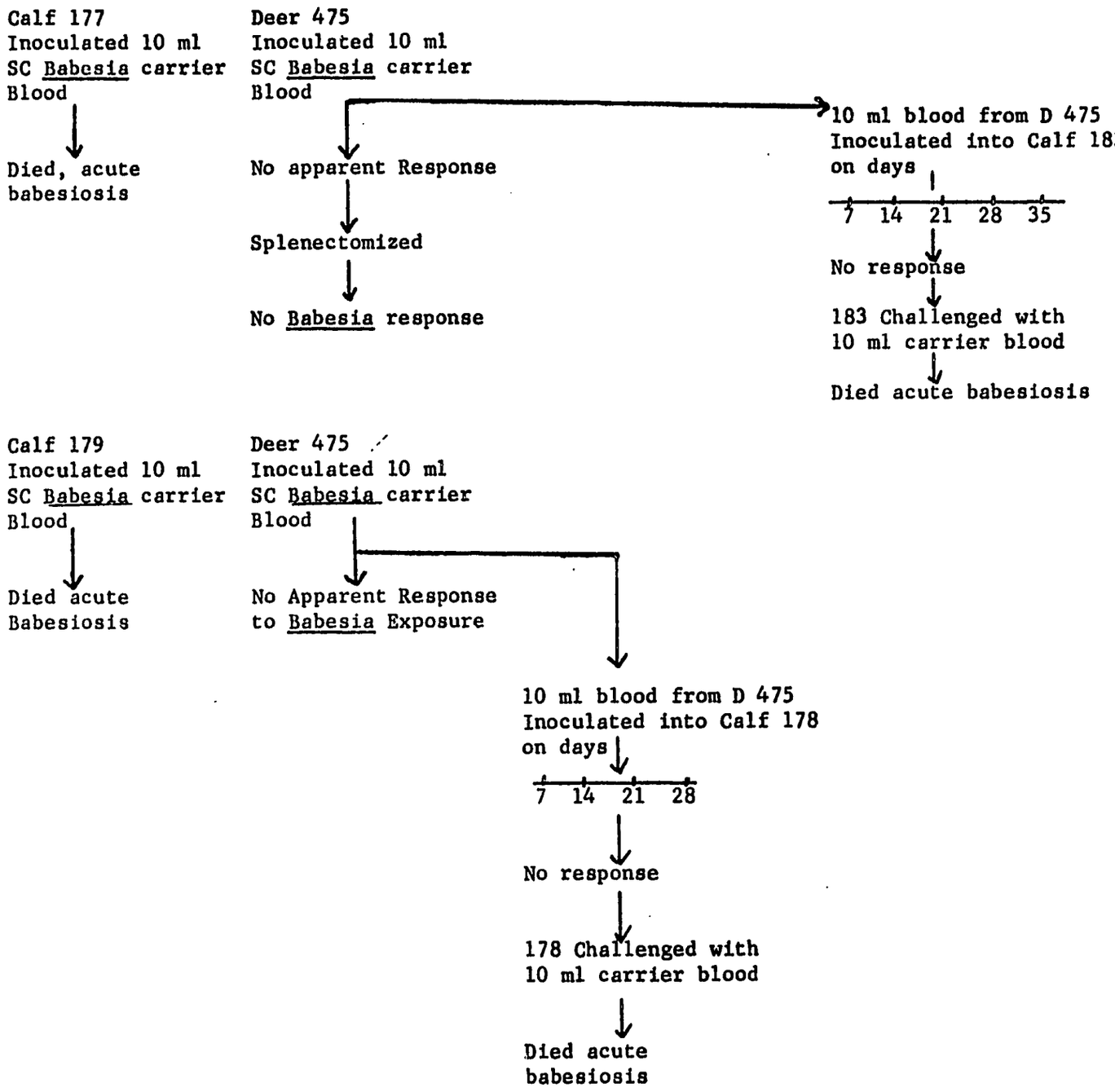
White-tailed deer, on the basis of limited trials, appear to be refractory to cattle Babesia, with no transmission occurring by either needle or tick means. Of equal significance was the observation that Babesia infected ticks, when undergoing feeding cycle (Larval - nymph - adult) on deer are then unable to transmit babesiosis to susceptible calves.

Boophilus ticks carried into the U.S. on deer are probably not infected with Babesia. If these ticks encounter Babesia carrier cattle in the U.S., then the possibility of so-called "hot ticks", or Babesia infected ticks transmitting infection becomes a real threat to the U.S. cattle population, but it is doubtful that tramsmission will occur in the absence of carrier cattle.

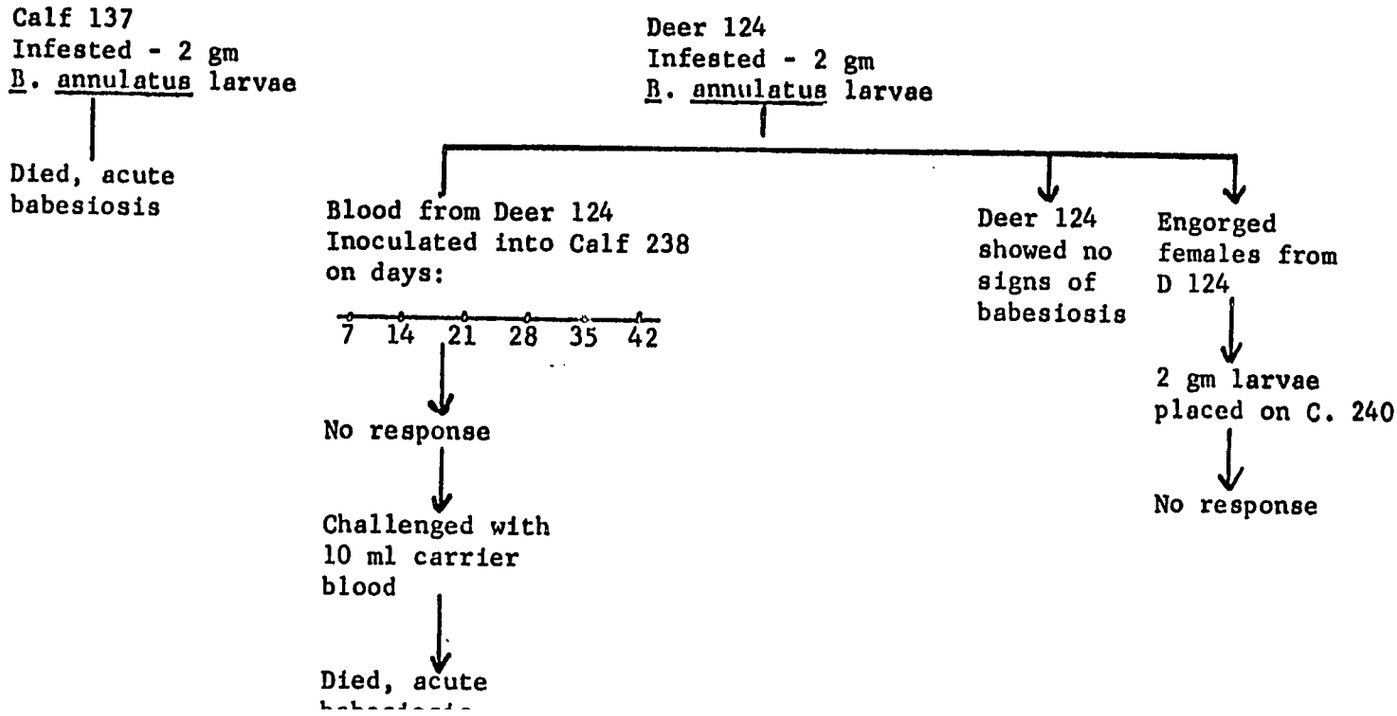
Efforts to Transmit *Babesia* to Deer 471



Efforts to Transmit *Babesia* to Deer 475



Efforts to Transmit *Babesia* to Deer 124



Babesia rodhaini Treatment #1

Therapeutic Studies with Ganaseg, 4A65, 356C61, and Oxytetracycline

The therapeutic value of Ganaseg[1] and 4A65[2] for *B. rodhaini* was recorded in the 1970 Annual Report. Because of the limited numbers, as well as limitations in the experimental design, it was felt that further studies of this nature were indicated. In conjunction with this study, 2 additional drugs, 356C61[3] and oxytetracycline, will be used. Neither of these drugs have been reported on with respect to their efficacy against *B. rodhaini*, even though both are highly effective against *Anaplasma*.

Materials and Methods:

A total of 50 white mice, approximately 32 gms in size, were infected with *B. rodhaini* by inoculating each with 0.5 ml, 10^{-3} dilution of blood showing a 47% parasitemia on day 1. On day 4, when mice were just beginning to show evidence of infection as evidenced by a low level parasitemia, treatment was initiated. The 50 mice were divided into 5 groups of 10 mice each. Four of these groups were treated, and 1 remained as a control. Group 1 was given 4 mg/kg Ganaseg; group 2, 4 mg/kg 4A65; group 3, 12 mg/kg oxytetracycline; and group 4, 5 mg/kg 356C61 on day 4. A second identical treatment was given each of the 40 mice on day 6. Thereafter, no further treatment was given. Individual parasitemias for each of the 50 mice was recorded on day 4,6,7 and 10. An analysis of variance was made to detect evidence of therapeutic efficacy.

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(1) Ganaseg: 4, 4' diamidine diaxoaminobenzene diaceturate

(2) 4A65: 3, 3'-Bis(2-imidazolin-2-yl)-carbanilide dihydrochloride

(3) 356C61: Gloxazone (a-ethoxy ethylglyoxal dithiosemicarbazone)

Results:

Low level parasitemias were noted in all groups 4 days after exposure (Table 16). On day 6, when a second treatment was administered, the influence of treatment was noted in the 4A65 group, which showed a marked regression of the parasitemia in contrast to the marked increase in parasitemia occurring in all other groups. At this time, the average parasitemia was somewhat lower in group 1, but the difference was not significant. On day 7, a further increase was observed in group 1, however, this increase was not nearly as severe as observed in groups 3, 4, and 5. An average parasitemia of 19% in group 1 was significantly lower than the parasitemias of 53% (group 3), 50% (group 4), and 57% (group 5). Group 2 (4A65) failed to show any detectable increase in parasitemia when compared to the previous day. On day 10 the parasitemia had dropped to 0.35% in group 1, and to 0% in group 2. Heavy death losses were observed in groups 3, 4, and 5 by day 10; and on day 12, 10 of 10 had died in group 3; 8 of 10 had died in group 4; and 10 of 10 had died in group 5. No death losses were observed in groups 1 or 2 that could be attributed to *Babesia* infections.

Table 16

The Effect of Treatment on Mice Infected with B. rodhaini

Treatment	No. of Animals	Day 4 Deaths	Day 4 Paras.	Day 6 Deaths	Day 6 Paras.	Day 7 Deaths	Day 7 Paras.	Day 10 Deaths	Day 10 Paras.	Day 12 Deaths
Group 1 Ganaseg	10	0	0.27	0	8.6	0	19.0	0	0.35	0
Group 2 4A65	10	0	0.25	0	0.08	0	0.08	0	0	0
Group 3 Oxytetracycline	10	0	0.14	0	11.3	0	53.0	6	56.0	10
Group 4 356C61	10	0	0.21	0	11.2	0	50.0	7	13.0	8
Group 5 Controls	10	0	0.22	0	14.8	0	57.0	9	17.0	10
Significance			NS		P<0.01		P<0.01		NT	
DRS					7.3		19.8*			

Treatment administered I.P on days 4 and 6 at the following levels:

Ganaseg: 4 mg/kg
4A65 : 4 mg/kg
Oxytetracycline: 12 mg/kg
356C61: 5 mg/kg

* Statistical evaluations were made excluding the 4A65 group.
DRS: Difference required for significance.
NS: Not significant
NT: No Test

Babesia rodhaini Treatment #2

The Effect of Treatment with Ganaseg and 4A65 on the *Babesia* Carrier

A total of 10 mice from group 1; 9 mice from group 2; and 2 mice from group 4 of the previous experiment (*B. rodhaini*, Treatment #1) were alive 34 days after the initial infection was induced. At this time none of these mice were showing evidence of *B. rodhaini* parasitemia. Group 1 had been treated 2 times at a 48-hour interval with Ganaseg at the rate of 4 mg/kg. 30 days previously. Group 2 had been treated 2 times at a 48-hour interval with 4A65 at the rate of 4 mg/kg, 30 days previously. Group 4 had been treated 2 times at 48-hour intervals with 356C61 at the rate of 5 mg/kg. 30 days previously.

On day 34, cardiac punctures were made from each of the 21 surviving mice, withdrawing 0.2 ml blood. This blood was then injected intraperitoneally (I.P.) in 0.1 ml amounts into each of 2 susceptible mice to determine the presence or absence of *B. rodhaini* in treated mice. In the process of making cardiac punctures, 2 of 10 mice died in group 1; 2 of 9 died in group 2; and 1 of 2 died in group 4.

To further evaluate the influence of infection and then treatment, the 16 mice of groups 1, 2, and 4, surviving cardiac punctures on day 34, were then challenged later the same day with 0.5 ml of a 10^{-2} dilution of blood having a PCV of 9% and a parasitemia of 91%.

Observations of PCV and parasitemia were made for 15 days after date of challenge.

Results:

The carrier status of treated mice and the effects of challenge of treated mice are recorded in Tables 17 and 18.

In group 1, following Ganaseg treatment, all mice were proven to be carriers. Of the 8 surviving cardiac puncture, 7 were solidly resistant to challenge. One animal, notwithstanding its carrier status, appeared to be susceptible to challenge. In group 2, none of the 9 mice treated with 4A65 were carriers of infection. Of the 7 surviving cardiac puncture, 4 were relatively resistant to challenge, whereas 3 were susceptible. The resistant mice of this groups failed to develop low PCVs or parasitemias of over 1.0%, however, 3 of the 4 resistant mice did show a parasitemia of less than 1.0%. In group 4, 1 of 2 surviving mice was a carrier, the other apparently free of infection. The 1 surviving mouse of this group (the carrier) was solidly resistant to challenge (Table 17).

The response of carrier and non-carrier mice to challenge is presented in Table 18. A total of 8 carrier mice, (1 from Group 4 and 7 from Group 1) showed an average low PCV of 40.8 $\pm$ 4.2, and an average high parasitemia of only 0.1%. The 1 carrier mouse in group 1 responding to challenge developed a low PCV of 18% which was accompanied by a high parasitemia of 29%. Recovery of this animal occurred rapidly after this parasitemia. The possibility that this response was actually a relapse infection cannot be ruled out, but the evidence is that the response was associated with challenge.

The response of non-carrier mice to challenge was more equally divided between those reacting and those not reacting to challenge. Of the 4 mice of group 2, apparently resistant to challenge, the average low PCV was 39.0 $\pm$ 3.2, whereas, the average high parasitemia was 0.45 $\pm$ .33. This

response is indicative of a marked sterile immunity. Even though this immunity was not complete, it was very evidently responsible for significant protection. No deaths occurred in this or the preceeding groups.

The susceptibility of 3 mice in this group accentuates the erratic nature of the sterile immunity. An average low PCV of 17.7% $\pm$ 1.6 was observed in this group, which was accompanied by an average high parasitemia of 25.3% $\pm$ 7.6. Two of the 3 mice in this group eventually died of acute babesiosis, suggesting little or no sterile immunity among these 3 mice.

Conclusion: (Treatment Experiments #1 and #2)

The superiority of 4A65 over Ganaseg in treating mice infected with *Babesia rodhaini* is clearly evident when these drugs are used in the dosage indicated in these experiments. Oxytetracycline was ineffective. It is very questionable that 356C61 was effective even though 2 of 10 mice treated with 356C61 survived, and 1 of these apparently lost the infection entirely. If the drug 356C61 has value against *B. rodhaini*, it is minimal, and of little or no practical value.

It is clinically significant that 2 treatments with 4A65 were 100% successful in eliminating the *Babesia* infection, whereas a similar treatment with Ganaseg resulted in 100% carriers. If these results are indicative of a similar action against cattle *Babesia*, the possibility of removing a carrier infection would be enhanced by the use of 4A65. This is not always desirable, so that care and judgement would be required in deciding the drug of choice for each particular situation.

The presence of a true sterile immunity, even though not entirely consistent, adds evidence to the belief that body defenses, against active Babesia infections, can be developed in the absence of carrier infections.

Table 17

Carrier Status and Susceptibility to Challenge of Treated Mice

	Number of Animals	No. Proven to be Carriers	No. Mice Surviving Cardiac Puncture	Result of Challenge	
				Resistant	Susceptible
Group 1	10	10	8	7	1
Group 2	9	0	7	4	3
Group 4	2	1	1	1	0

Table 18

Challenge Results on Mice Previously Infected and then Treated.

	Group	No. of Animals	Average low PCV	Average high Parasitemia	Deaths due to Challenge
Carriers Resistant to Challenge	Gr. 1 Gr. 4	7 1	40.8 ±4.2	0.10 ± 0.18	0
Carriers Susceptible to Challenge	Gr. 1	1	18	29	0
Non-Carriers Resistant to Challenge	Gr. 2	4	39.0 ± 3.2	0.45 ± .33	0
Non-Carriers Susceptible to Challenge	Gr. 2	3	17.7 ± 1.6	25.3 ± 7.6	2

B. rodhaini Treatment #3

Drug Prophylaxis Against B. rodhaini in Mice

Part 1

In the 1970 Annual Report, Dr. R.A. Todorovic suggested a prophylactic value for the drug 4A65 when administered to cattle prior to exposure. This experiment was designed to measure this response and compare 4A65 with Ganaseg in the role of preventive drug, using B. rodhaini as a model.

Mice will be treated with 4A65 and Ganaseg on day 0 and then challenged by blood inoculation on days 0-4-7-14 and 21. A total of 25 mice were injected I.P. with 4A65 at a dose rate of 2 mg/kg. A similar number was injected I.P. with 4 mg/kg Ganaseg. On day 0, 5 mice injected with 4A65, 5 mice injected with Ganaseg, and 5 untreated control mice were each challenged with 0.5 ml, 10^{-3} dilution of blood showing a 50% parasitemia. The challenge inoculum for days 4-7-14 and 21 was identical except for the parasitemia of the infected blood, and this was 45%, 42%, 38% and 52% respectively.

Observations on each of the challenged mice consisting of PCVs and parasitemia were made 2 times a week.

The results are recorded in Table 19. Mice challenged on the same day of treatment showed a marked influence to the drug on the course of infection. No deaths occurred in the 4A65 group even though 2 mice reacted. One death occurred in the Ganaseg group, and all reacted, however, parasitemias were lower than seen in the controls. There was a difference in average incubation time as determined by parasitemia with 4A65 being the longest and the control the shortest. Challenge at day 4, resulted in a Babesia

response in all mice. A considerably longer incubation time was noted for the 4A65 group along with lower parasitemias. Three mice died in each of the 2 treated groups, whereas, all 5 controls died. Challenge at day 7 produced very little difference in animal response between groups. All mice reacted in approximately the same length of time with peak parasitemias occurring on the 11th day. In the 4A65 group 4 of 5 died, and in the other groups all mice died. Challenge on days 14 and 21 resulted in 100% response with little or no difference occurring in the incubation time or the severity of reaction between groups.

Conclusion:

Little or no prophylactic activity beyond 4 days was noted as the result of treatment with either 4A65 or Ganaseg. These drugs, in the dosage used, did not persist in sufficient quantity to influence the course of infection and seem to be of questionable value for use in this manner.

Table 19

Prophylactic Effects of 4A65 and Ganaseg When Challenge Was Administered at Varying Intervals After Treatment

	Days After Challenge								
	4	7	11	14	18	22	25	29	32
Challenge:Day 0									
4A65:Deaths	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Parasitemia (%)	0	0	1	0	2	7	0	0	-
Ganaseg:Deaths	0/5	0/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5
Parasitemia (%)	0	16	12	1	1	.1	7	.1	-
Control:Deaths	0/5	1/5	5/5	X	X	X	X	X	X
Parasitemia	2	60	-	-	-	-	-	-	-
Challenge:Day 4									
4A65;Deaths	0/5	0/5	0/5	0/5	2/5	3/5	3/5	3/5	3/5
Parasitemia	0	0	0	2	22	0	0	0	0
Ganaseg:Deaths	0/5	0/5	3/5	3/5	3/5	3/5	3/5	3/5	3/5
Parasitemia	1	62	21	1	0	0	0	0	0
Control:Deaths	0/5	1/5	5/5	X	X	X	X	X	X
Parasitemia	1	66	-	-	-	-	-	-	-
Challenge:Day 7									
4A65:Deaths	0/5	0/5	3/5	4/5	4/5	4/5	4/5	4/5	4/5
Parasitemia	0	27	50	0	0	0	0	0	0
Ganaseg:Deaths	0/5	0/5	4/5	5/5	X	X	X	X	X
Parasitemia	0	25	72	-	-	-	-	-	-
Control:Deaths	0/5	1/5	4/5	4/5	4/5	X	X	X	X
Parasitemia	-	28	2	10	-	-	-	-	-

Table 19 (continued)

Prophylactic Effects of 4A65 and Ganaseg When Challenge Was Administered at Varying Intervals After Treatment

	Days After Challenge								
	4	7	11	14	18	22	25	29	32
Challenge:Day 14									
4A65:Deaths	0/5	0/5	4/5	4/5	5/5	X	X	X	X
Parasitemia	0	53	28	5	-	-	-	-	-
Ganaseg:Deaths	0/5	0/5	3/5	4/5	4/5	4/5	4/5	4/5	4/5
Parasitemia	0	64	35	13	-	-	-	-	-
Control:Deaths	0/5	0/5	2/5	5/5	X	X	X	X	X
Parasitemia	0	34	63	-	-	-	-	-	-
Challenge:Day 21									
4A65:Deaths	0/5	0/5	5/5	X	X	X	X	X	X
Parasitemia	.2	74	-	-	-	-	-	-	-
Ganaseg:Deaths	0/5	2/5	5/5	X	X	X	X	X	X
Parasitemia	.5	79	-	-	-	-	-	-	-
Control:Deaths	0/5	2/5	5/5	X	X	X	X	X	X
Parasitemia	.3	80	-	-	-	-	-	-	-

Drug Prophylaxis Against *B. rodhaini* in Mice

Part 2

In part 1, a single drug level was used, varying the challenge time. This experiment varied the dose level for 4A65 and Ganaseg, with a challenge administered to all mice 7 days after treatment.

The drug 4A65 was injected I.P. at the levels of 2-4-8-16 and 32 mg/kg. Five mice were used at each drug level. Seven days after drug inoculation all mice were challenged with 0.5 ml I.P. of a 10^{-3} dilution of blood showing a 70% parasitemia and a 26% PCV.

Ganaseg was injected I.P. at the levels of 2-4-8-16-32 and 64 mg/kg. Five mice were used at each of the drug levels except 64 mg. and 4 mice were used in this instance. The same challenge inoculum was administered at the same interval as described for 4A65.

Parasitemias on thin Giemsa-stained blood smears were made twice weekly for a period of 43 days after challenge.

Results:

Ganaseg, when administered I.P. in the levels of 2-4-8-16 and 32 mg/kg, had no demonstrable influence on the course of infection when challenge was administered 7 days after treatment. Ganaseg administered at the level of 64 mg/kg prevented death loss in all 4 mice challenged 7 days after treatment. A characteristic high parasitemia was noted in this group at day 7 and day 21, but all mice recovered.

A marked prophylactic effect was detected in all groups receiving 4 mg or more 4A65, when compared to infections occurring in the control and Ganaseg groups. The administration of 2 mg/kg produced the least influence, but the average parasitemias were lower than seen in the Ganaseg group, even though 4 of 5 mice died of babesiosis. It is interesting to note that the 7-day challenge recorded in part 1 also resulted in 4/5 deaths. The losses occurring in mice receiving 4-8 and 16 mg were markedly less than seen with the Ganaseg or control group. No losses occurred in the group receiving 32 mg/kg.

Conclusions:

Ganaseg was ineffective as a chemical prophylactic agent against *B. rodhaini*. The drug 4A65, however, demonstrated considerable influence on the challenge inoculum at 4-8-16 and 32 mg/kg levels when the challenge was given 7 days after treatment. The duration of this activity must now be ascertained.

Table 20

Prophylactic Effects of 4A65 and Ganaseg When Used in Varying Dose Levels Followed by a 7-Day Challenge

	Days After Challenge										
	1	4	7	10	14	17	21	24	28	35	39
Ganaseg											
2 mg/kg: Deaths	0/5	0/5	0/5	5/5	X	X	X	X	X	X	X
Parasitemia	0	.9	68	-	-	-	-	-	-	-	-
4 mg/kg: Deaths	0/5	0/5	1/5	4/5	5/5	X	X	X	X	X	X
Parasitemia	0	0	74	5	-	-	-	-	-	-	-
8 mg/kg: Deaths	0/5	0/5	0/5	5/5	X	X	X	X	X	X	X
Parasitemia	0	.1	72	-	-	-	-	-	-	-	-
16 mg/kg: Deaths	0/5	0/5	0/5	4/5	5/5	X	X	X	X	X	X
Parasitemia	0	0	76	40	-	-	-	-	-	-	-
32 mg/kg: Deaths	0/5	0/5	0/5	4/5	5/5	X	X	X	X	X	X
Parasitemia	0	0	71	10	-	-	-	-	-	-	-
64 mg/kg: Deaths	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Parasitemia	0	2	30	12	1	.1	9	.1	0	.5	6

Table 20 (continued)

Prophylactic Effects of 4A65 and Ganaseg When Used in Varying Dose Levels Followed by a 7-Day Challenge

	Days After Challenge										
	1	4	7	10	14	17	21	24	28	35	39
4A65											
2 mg/kg: Deaths	0/5	0/5	0/5	1/5	4/5	4/5	4/5	4/5	4/5	4/5	4/5
Parasitemia	0	4	10	28	0	0	.8	.1	.1	0	0
4 mg/kg: Deaths	0/5	0/5	0/5	0/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5
Parasitemia	0	4	.9	28	28	.4	.5	3	.1	.5	.5
8 mg/kg: Deaths	0/5	0/5	0/5	0/5	0/5	0/5	1/5	2/5	2/5	2/5	2/5
Parasitemia	0	3	0	1	7	32	11	0	1	1	2
16 mg/kg: Deaths	0/5	0/5	0/5	0/5	0/5	0/5	1/5	1/5	1/5	1/5	1/5
Parasitemia	0	5	0	0	.4	14	0	5	1	.4	1.5
32 mg/kg: Deaths	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Parasitemia	0	2	0	0	0	0	13	.2	0	0	0
No Treatment											
Control: Deaths	0/5	0/5	0/5	5/5	X	X	X	X	X	X	X
Parasitemia	0	5	71	-	-	-	-	-	-	-	-

Babesia rodhaini

Duration of Immunity Following Premunition

In a previous study evidence has been presented to indicate that a predominately sterile immunity occurred following infection and treatment with 4A65, and carrier immunity occurred following infection and treatment with Ganaseg.

The principle purpose of this experiment was to measure the duration of immunity following infection and treatment with 4A65 and Ganaseg. A secondary object was to measure the type immunity produced, whether sterile or carrier, and to compare the degree and duration of each type. As a third objective, observations and data will be recorded to evaluate the 2 drugs as therapeutic agents.

Materials and Methods:

On day 0, 50 mice were inoculated I.P. with 0.5 ml of a 10^{-3} dilution of infected blood having a 83% parasitemia. On day 4 and again on day 7 all mice were treated with 4A65 at the rate of 2 mg/kg injected I.P. These mice were then divided at random into 10 cages with 5 mice per cage and labeled pen 11, 12, 13 ... 20.

In parallel with the above, an additional 50 mice were similarly infected on day 0 and treated on days 4 and 7 with Ganaseg at the rate of 4 mg/kg I.P. These mice were placed in cages numbered pen 21, 22, ... 30.

On days 14, 28, 42, 56, 84, 112, and 140 groups of 5 mice were selected from each of the 2 treatments and checked for evidence of infectivity. This was done by withdrawing 0.2 ml blood by cardiac puncture and inoculating 2 mice I.P. each with 0.1 ml. The mice in each group surviving the cardiac puncture were then challenged by inoculating I.P., 0.5 ml 10^{-3} dilution of blood showing a 30 to 60% parasitemia. Because of mortality in the Ganaseg group due to B. rodhaini and losses associated with cardiac punctures, the full 50 mice from each group were not available for use, and experiment was terminated at day 140.

Following the initial exposure and treatment observations consisting of PCV and parasitemias were made twice weekly on 1 mouse from each of 10 pens in each group to establish a pattern of response to exposure and treatment. Following challenge, similar observations were made twice weekly for 40 days to measure animal response.

Results:

The response of 10 mice, infected with B. rodhaini, treated with 4A65 and Ganaseg is presented in Figures 28 and 29. These results are consistent with past results, showing a disappearance of parasitemia soon after the second 4A65 treatment. Mice treated with Ganaseg showed a more prolonged and higher parasitemia accompanied by some death losses. Of the 50 Ganaseg treated animals, 3 died on day 7, 7 died on day 11 and 1 died on day 14. No deaths occurred among the 4A65 treated group. A low level parasitemia was noted in the Ganaseg group throughout the entire observation period.

The results of tests for carrier status and resistance to challenge are presented in Table 21. On day 14 all mice treated with Ganaseg were found to be B. rodhaini carriers. No carriers were seen among the 4A65 group. In the 4A65 group, 4 mice responded to challenge, with 1 death, but no response to challenge was seen in the Ganaseg group.

On day 28, 2 mice were found to be carriers in the 4A65 group, and 3 were free of infection. All mice were carriers in the Ganaseg group. Discounting deaths from cardiac punctures, all the 4A65 treated mice responded to challenge, however, no deaths occurred. There was no response to challenge in the Ganaseg group.

On day 42 the pattern persited in that all mice in the Ganaseg group were carriers, and the 4A65 groups were all free of infection. Among the 4A65 animals, all the surviving mice (4) responded to challenge, however, no deaths occurred. Of the 4 surviving mice in the Ganaseg group, 2 apparently responded to challenge, but very mildly, and some question occurs as to whether or not this may have been a relapsing type infection. As on the previous challenge dates (day 14 and 28), all the controls developed high parasitemias, and eventually died.

On day 56, 2 of the 5 mice checked in the Ganaseg groups were free of the carrier status. The remaining animals were carriers. Of the 5 in the 4A65 group, 4 were free of infection and 1 showed a carrier infection. All mice surviving cardiac puncture in the 4A65 group responded to challenge, whereas only 1 mouse of the 3 surviving in the Ganaseg group showed evidence of challenge response. No deaths were observed in either of the treated groups, in contrast to the 100% mortality in the controls.

On day 84 the continued disappearance of the carrier status in the Ganaseg group was obvious. Infection could not be recovered in 3 of the 5 mice tested. No infection was recorded in the 4A65 group. All of the mice in the 4A65 group responded to challenge with 2 of this number dying. The developing parasitemia was as great in the 4A65 group as the controls. All the controls died, so that it is safe to assume that a residual, sterile immunity was persisting, but at a lower level. Only 1 of the Ganaseg mice responded to challenge, and this animal survived. A sterile immunity is now showing up in this group, which, as expected, is of greater intensity than in the 4A65 group.

On day 112 no carrier infections were detected in either of the 2 groups. The residual, sterile immunity was probably higher in the Ganaseg group since only 3 of 5 showed a response to challenge as compared to 5 of 5 responding to challenge in the 4A65 group. In addition to this, the developing parasitemia was higher in the 4A65 group. In contrast to this, however, 2 of the Ganaseg group died, and none in the 4A65 group died. This observation points out the wide individual variation that occurs in mice infected with B. rodhaini. It would appear that a residual immunity is still persistent in each of the treated groups since all the controls died.

On day 140 no carrier infections were noted, and the response to challenge followed a similar pattern in that 4 of 5 in the 4A65 groups responded, and 1 of 2 in the Ganaseg group responded. Parasitemias were high in the treated groups, with 3 dying in the 4A65 group and 1 of 2 dying in the Ganaseg group. Except for a slightly reduced mortality, as compared to the controls, very little evidence of residual immunity was apparent. An immunity apparently does exist, but in low levels.

Results are tabulated in Table 22 to further summarize the response to challenge of those mice surviving cardiac puncture. Of 29 mice in the 4A65 group, 27 were free of infection and 2 were carriers. Of the 27 free of infection, 93% responded to challenge, with 6 (22%) deaths due to infection. Of 28 mice in the Ganaseg group, 11 (39%) were free of infection and 17 (61%) were carriers of infection. Of the 11 free of infection, 45% responded to challenge as compared to 24% responding to challenge among the carrier animals. There were 3 deaths occurring following the challenge of the group free of infection.

Conclusions:

Carrier infection while occurring in nearly all mice treated with Ganaseg does not persist past the 112th day. This period of time is sufficiently long to rule out a long term drug effect and probably represents an ability on the part of the host to rid itself spontaneously of the infection. As this occurs, the immunity begins to wain, but does not totally disappear up to 140 days. During the carrier phase, immunity or protection from fatal babesiosis is almost complete, but when a so-called sterile immunity takes over, the immune position of the mouse is less certain.

A fairly high level of sterile immunity was observed in the 4A65 group up to 56 days, at which time a high average parasitemia was observed following challenge, but was not accompanied by death loss. Thereafter, this immunity dropped rapidly. Some evidence of protection apparently persisted for as long as 112 days.

The carrier state quite obviously provides a more solid protection against heavy challenge, however, in producing this type of immunity losses due to infection were encountered. A sterile immunity is reasonably short acting, and is not as reliable as the carrier type. Carrier animals, when the infection is lost, apparently proceed with a sterile immunity for a period of time before becoming susceptible to a challenge exposure, however, the duration of this immunity is not known.

Table 21

Babesia rodhaini Premunition Using 4A65 and Ganaseg

	Day 14			Day 28			Day 42			Day 56		
	Pen 11 4A65	Pen 21 Gan.	Cont.	Pen 12 4A65	Pen 22 Gan.	Cont.	Pen 13 4A65	Pen 23 Gan.	Cont.	Pen 14 4A65	Pen 24 Gan.	Cont.
Sterile/carrier	5/0	0/5	---	3/2	0/5	---	5/0	0/5	---	4/1	2/3	---
Death due to cardiac puncture	0	0	---	3	1	---	1	1	---	2	2	---
No. responding to challenge	4	0	5	2	0	5	4	2	5	3	1	5
Avg. incubation time - days	12	---	7.2	16	---	4	4	15	4	8	11	4.8
Avg. high parastiemia (%)	9.2	.34*	60.0	5.7	---	33.4	8.5	---.0	81.2	38	25	73.6
No. of deaths	1	0	5	0	0	5	0	0	5	0	0	5
Avg. time of death (days)	15	---	13.4	---	---	12	---	---	11	---	---	11

*Relapsing Parasitemia apparently not due to challenge

Sterile: Refers to the absence of a recoverable *Babesia* infection at the time of challenge.

Carrier: Mice in which *Babesia* was recovered at the time of challenge.

Table 21 (continued)

Babesia rodhaini Premunition Using 4A65 and Ganaseg

	Day 84			Day 112			Day 140		
	Pen 15 4A65	Pen 25 Gan.	Cont.	Pen 16 4A65	Pen 26 Gan.	Cont.	Pen 17 4A65	Pen 27 Gan.	Cont.
Sterile/carrier	5/0	3/2	---	5/0	5/0	---	5/0	2/0	---
Death due to cardiac puncture	0	0	---	0	0	---	0	0	---
No. responding to challenge	5	1	5	5	3	5	4	1	5
Avg. incubation time - days	5.6	15	8	8.8	6.0	3.0	5.8	5	5
Avg. high parasitemia (%)	49	8	32	26.2	16	69	47	71	33
No. of deaths	2	0	5	0	2	5	3	1	5
Avg. time of death (days)	13	---	13.4	---	20	11	10	12	8

Sterile: Refers to the absence of a recoverable *Babesia* infection at the time of challenge.

Carrier: Mice in which *Babesia* was recovered at the time of challenge.

Figure 28

Response to Treatment with 4A65
(Exposed on Day 0)

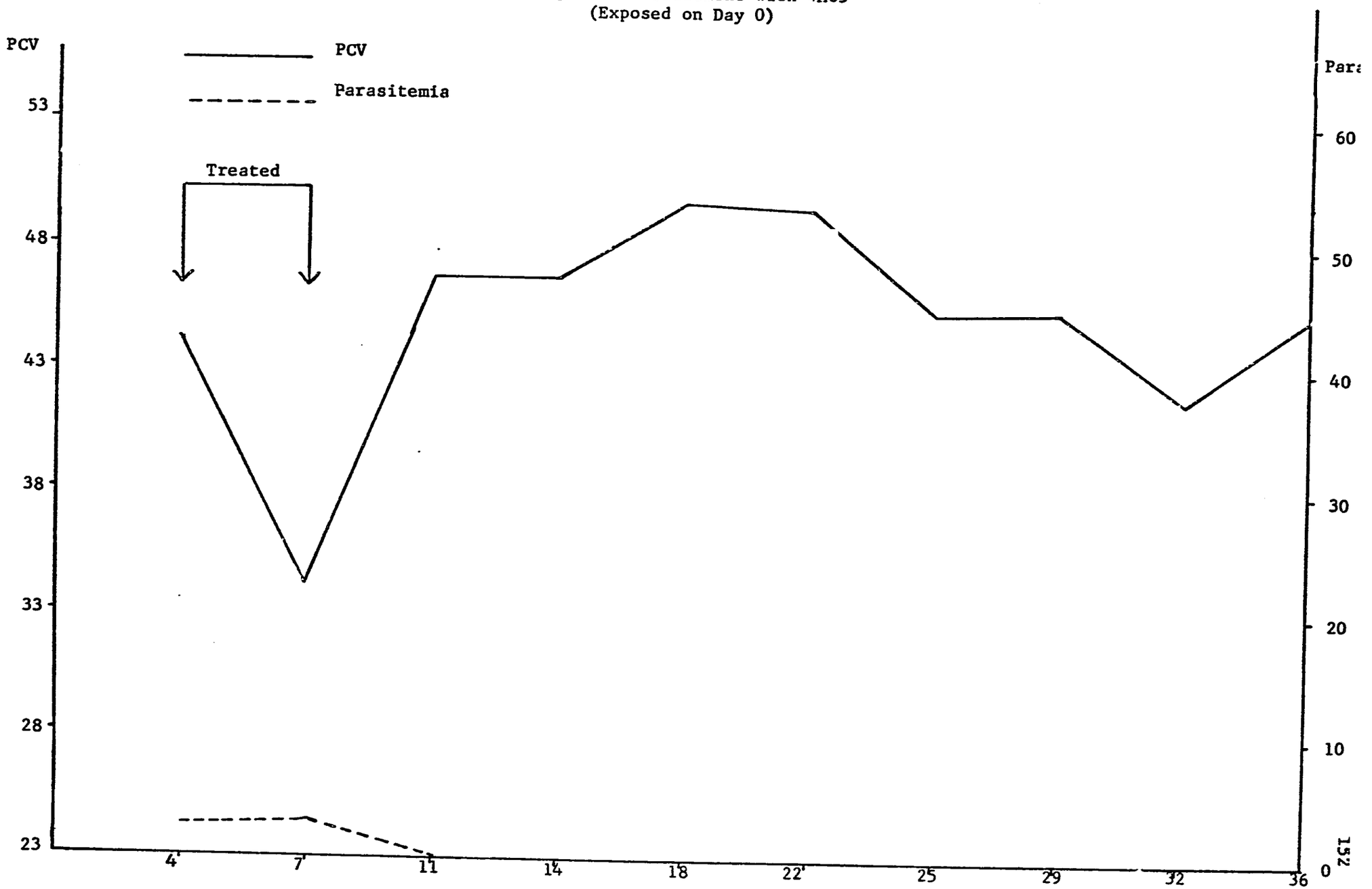


Figure 29

Response to Treatment with Ganaseg
(Exposed on Day 0)

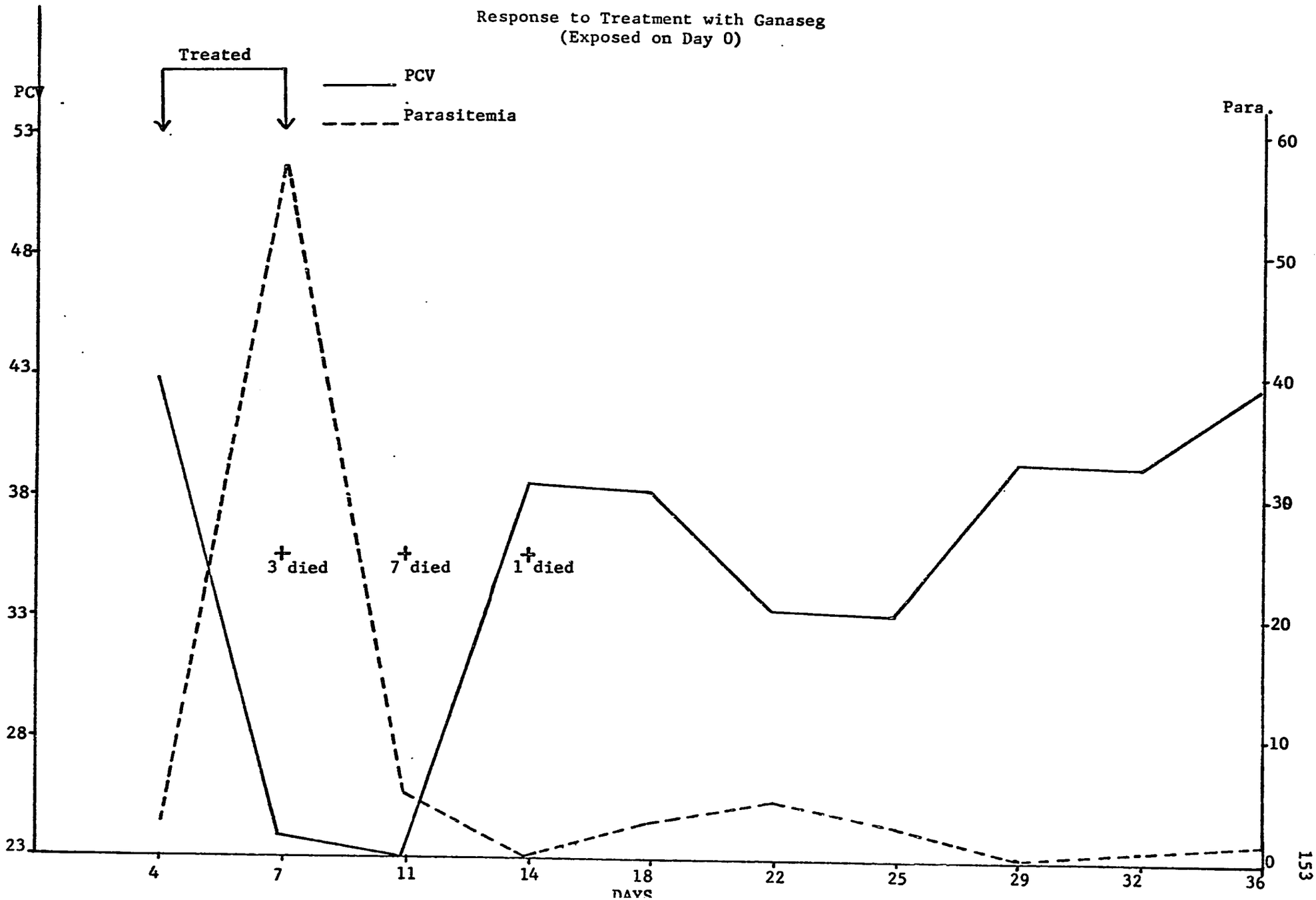


Table 22

Carrier Status of Mice at the Time of Challenge and the Response to Challenge Over a 140 Day Period

Group	Type of Immunity	No. of Mice	Response to Chall. (%)	Mortality (%)
4A65	Sterile	27 (93%)	93%	6 (22%)
	Carrier	2 (7%)	100%	0
Ganaseg	Sterile	11 (39%)	45%	3 (27%)
	Carrier	17 (61%)	24%	0

Sterile: Refers to the absence of a recoverable *Babesia* infection at the time of challenge.

Carrier: Mice in which *Babesia* was recovered at the time of challenge.

Babesia rodhaini Pathogenesis #1

Relationship of PCV and Parasitemia

In many instances, babesiosis in mice, produced by *B. rodhaini*, is so acute that death sometimes occurs before a low PCV is noted, or even before a high parasitemia is seen. A similar pattern has been noted to a lesser extent in cattle *Babesias*, where the disease is so acute that the association of parasitemia and anemia is not particularly obvious. Schroeder and Ristic, in reviewing the relationship between parasitemia and anemia, have concluded that the anemic response is not necessarily related to parasitemia. They state that "anemia occurs between the 9th and 11 day ... irrespective of the degree of parasitemia" (p. 64, vol. 1 Infectious Blood Diseases of Man and Animals).

Our experience has been that many factors may influence the course of infection. The incubation time can be prolonged (as reported in the 1970 annual report) by inoculums of low infectivity. These infections appear to be accompanied by a higher percentage of surviving mice than when a massive challenge is administered.

The treatment experiments in which 4 drugs (4A65, 356C61, Ganaseg and oxytetracycline) were tried, along with controls, resulted in a wide range of parasitemias, and since PCVs had been conducted on all animals, the opportunity presented itself to conduct a correlation analysis to determine if a significant relationship between parasitemia and PCV could be identified by statistical methods.

Parasitemias and PCV determinations were conducted on 40 mice. Parasitemias were determined by counting RBCs and then determining the percent of these cells that were parasitized as seen on Geimsa-stained, thin blood smears. PCVs were performed using a micro-hematocrit centrifuge. Blood was obtained from the tail by clipping a small portion, each time a blood sample was required, filling the hematocrit (heparinized) capillary tube, and making the slide from the blood as it came from the tail.

The PCV range of these 40 mice was 19% to 41% with an average of 30.5%. The parasitemia range was 1% to 81% with an average of 44.8%. The regression coefficient (b) was -2.3 with a drop in PCV associated with an increase in parasitemia. This value was highly significant with a t value of 5.47. The correlation coefficient (r) was 0.663, and with 38 degrees of freedom is highly significant ($P<0.01$).

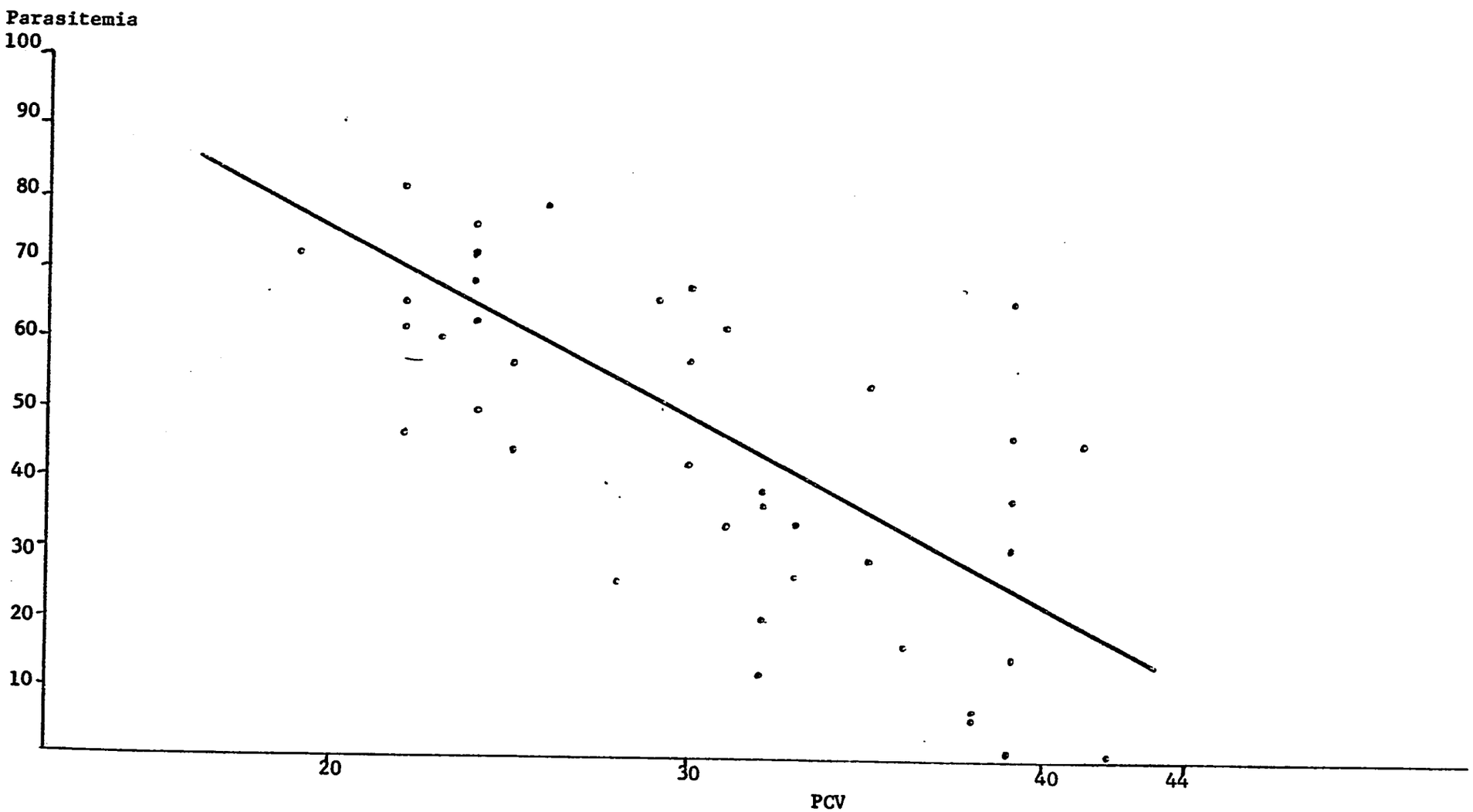
The slope and points are presented in figure 27.

Conclusion:

A significant relationship between PCV and parasitemia has been demonstrated by statistical means. Individual variations are such that similar conclusions might not be valid on smaller numbers. In all probability a short lag time does occur in this relationship, in that the parasitemia preceeds the anemia, but this time factor is not sufficiently great to offset the major relationship and interdependence of parasitemia and anemia.

Figure 27

Linear Relationship of PCV and Parasitemia
in *B. rodhaini* infections in mice



Babesia rodhaini Pathogenesis #2

Comparative Blood and Spleen Titers

The small quantity of blood available from a mouse acutely infected with *B. rodhaini* creates problems when a quantity of infectious material is desirable. For this reason, consideration has been given to other tissues that might be suitable sources of antigenic material. In view of experience with other hemotropic agents, it seems probable that the spleen might be a good source of infectious material. A degree of splenomegalia has been encountered with *B. rodhaini* infections. *Babesia* infections are accompanied by an average spleen size of 500 mg as opposed to 176 mg for normal spleen (1970 annual report).

A comparative titration was, therefore, made of blood and spleen from a single *B. rodhaini* infected mouse. Blood was diluted in a phosphate buffer saline solution (PBS) with a pH of 7.2 to give final dilutionsof $10^{-4,5,6,7,8,}$ and 9. Five mice were each injected I.P. with 0.5 ml of each dilution. Spleen was weighed and prepared as a 1:1000 dilution in PBS, then ground in a Vir-tis tissue grinder 1 minute at medium speed. The resulting material was then diluted in 10 fold steps and titrated as described for blood. To cover the possibility that grinding might influence the titer of this material, a 1:1000 dilution of blood was similarly ground, diluted, and titrated.

The following results were obtained:

Unground blood	$10^{-8.65}$
Ground(Vir-tis) blood	$10^{-7.67}$
Spleen	$10^{-7.0}$

Conclusion:

Splenic material is a good source of infectious material. While not so high a titer as blood in acute babesiosis, it is highly infectious. Grinding of infected blood in a Vir-tis was responsible for almost a log drop in titer. When this factor is considered, the spleen is not much lower than blood in the number of infectious units present.

B. rodhaini Adjuvant Vaccine Experiment

Success in the production of an adjuvant vaccine for anaplasmosis, and the apparent development of immunity without the persistence of infection, led us to a preliminary investigation of the possibilities of an adjuvant Babesia vaccine. The B. rodhaini model appeared suited for this work, since reasonably large numbers of mice were available, and the infectious organism produced a consistent and predictable reaction in susceptible mice.

For this experiment 8 antigens were employed, each being tested in 10 mice. The vaccine injections were given in 0.1 ml amounts intraperitoneally (I.P.) on day 0 and on day 28. Challenge consisting of 0.5 ml of a 10^{-2} dilution of infected blood having a parasitemia of 86% was given half the vaccinated mice on day 59. The remaining mice were similarly challenged on day 101, with a 10^{-3} dilution of blood having a parasitemia of 68%.

Two antigen series were prepared, BR and BRV. The BR series was prepared from 50 mice that had been exposed 8 days previous by the usual 10^{-3} dilution, injected in the amount of 0.5 ml I.P. These mice were showing high parasitemias (over 50%) at the time of harvest. Blood was collected by cardiac puncture, using heparin as an anti-coagulant. Approximately 28 ml of blood was harvested. The RBCs were washed 1 time in VB, and 7 ml packed cells recovered. At the same time blood was harvested, spleens were collected and ground in VB using a Vir-tis tissue homogenizer ("45") to give a final 5% suspension. This was frozen until used.

The BR blood antigens (BR-1, 2 and 3) were prepared by lysing the packed RBCs in the 10 volumes of distilled water and washing 1 time in VB using 35,000 x G, 40 minutes for each spin. As described for similar Anaplasma antigens a light weight fluffy sediment was observed overlaying a dark, dense button. These two sediments were seperated. The heavy sediment was prepared as a 2% suspension in VB by adding 27.6 ml to 0.564 gms, and sonicated 3 minutes at 80 watts. This was labeled BR #1. The light weight sediment was prepared as a 5% suspension in VB, sonicated, labeled BR #2, and frozen.

The supernate following centrifugation of lysed cells was collected. The pH was reduced to 5.0 by the addition of .1 N HCl, and allowed to stand overnight. The precipitate was then collected by centrifugation 40 minutes at 35,000 x G. A sediment weighing 0.191 gms was then suspended in 18.9 ml VB to give a 1% suspension, labeled BR #3, and frozen until used.

A 5% spleen susp[illegible]n in VB was labeled BR #4.

The BRV series of antigens were prepared from 64 acutely infected mice having an average parasitemia of 62.6 $\pm$ 8. Approximately 32 ml of blood was collected by cardiac puncture using heparin as an anticoagulant. The spleens were also harvested. The blood was centrifuged and washed 1 time using 650 x G for 10 minutes. These cells were then lysed with 10 volumes of distilled water.

The lysed cells, after standing overnight, were centrifuged 40 minutes at 35,000 x G. The resulting sediment was washed 1 time in distilled water, using the same centrifuge setting. The sediments layered out as

described for the BR series, but no effort was made to seperate them. The total sediment weight was 2.64 gms, which was mixed with 63.4 ml VB to give a 4% suspension. This suspension was ground at high speed in the Vir-tis and labeled BRV #1. The supernate following RBC lysis and centrifugation was treated with 1 N HCl to give a pH of 4.8. This material, after standing overnight at 5°C, was centrifuged 40 minutes at 35,000 x G. A final precipitate, weighing 1.717 gms, was recovered and resuspended in 41.2 ml VB to give a 4% suspension, labeled BRV #2 and frozen at -20°C until used.

The 64 spleens weighed 28.1466 grams. A 20% suspension was prepared by adding 113 ml VB and mixing at medium speeds on the Vir-tis (tissue homogenizer). This material was centrifuged 10 minutes at 800 rpm (approximately 110 x G). The supernate was saved. The sediment was reground in the Vir-tis using VB, and again centrifuged 800 RPM for 10 minutes. The sediment following this step was discarded. The 2 supernates were pooled and centrifuged 40 minutes at 35,000 x G. The resulting sediment was washed in VB, mixing with the Vir-tis at moderate speeds, and again centrifuged 40 minutes at 35,000 x G. The sediment was layered. An attempt was made to separate an upper and lower sediment. Even though some difficulty was encountered, 3.756 gms was obtained from the top, which was suspended in 71.37 ml VB with mixing at full speed on the Vir-tis tissue homogenizer to give a final 5% suspension. The remaining sediment weighed 4.6 gms. A 5% suspension was prepared in the same manner by adding 87.45 ml VB. The first or light sediment was designated BRV #3, and the second BRV #4.

The 8 antigens were mixed with adjuvants to give the final vaccine preparation as follows:

Antigen	2.5 ml.
Mineral Oil containing 2 mg *Mycobacteria butyricum*/ml.	2.0 ml.
Arlacel "A"	0.5 ml.
Pen-Strep (20,000 U. Pen,25 mg. strep)	0.1 ml.

An emulsion was formed by sonication for about 30 seconds, or until a good emulsion appeared. Over sonication resulted in excessive thickness making the material extremely difficult to work with.

A control using veronal buffer in place of antigen was prepared, and 10 mice were inoculated with this material in the same way as used for vaccinated mice. Vaccination consisted of inoculating 0.1 ml of the antigen emulsion I.P. in each mouse on day 0, and again on day 28, with challenge schedules as previously mentioned.

Results:

Of the 90 mice vaccinated 87% survived to receive challenge inoculums. The occasional death was observed, showing no specific pattern, the cause being unknown. The numbers challenged, and the result of challenge are given in Tables 22 and 23. In addition to the VB antigen control, 5 untreated, susceptible mice were used. Significant differences were observed in the percent parasitemia at both the 59 and 101 day challenge. Of interest, however, was the lack of large, significant differences between vaccine groups and the VB vaccine group. Vaccination with VB plus the adjuvants produced as much protection as did most of the antigens. When compared to the untreated controls most vaccinated mice including the

VB controls showed marked protection. The act of inoculating mice I.P. resulted in significant interference with B. rodhaini's ability to grow, produce a high parasitemia, and cause death. BR #1 gave every indication of having produced a degree of specific protection over and above the interference noted elsewhere. On the 59-day challenge only 1 of 5 reacted, with an average high parasitemia of 0.6%. BRV #1, a similar antigen, gave evidence of being the next most active. Beyond these 2 antigens it is difficult to ascribe any degree of protection to the other preparations. The mechanism by which water, salts, and oil adjuvants would retard B. rodhaini multiplication is unknown.

Conclusion:

The observance of non-specific protection suggests a possibility that we may have an inteferon system working to prevent serious infection, based solely on the non-specific action of the vaccine inoculum. Evidence does exist however that BR #1 and possibly BRV #1 are immunologically active antigens. This experiment has been conducted 2 times, the first is not included in the report. During the first experiment the absence of reactions in the control (VB control) led us to the conclusion that mice had been inadvertantly mixed up when cages were cleaned, therefore, it was not pursued, and a second effort was made. Caution was used to avoid the supposed problem a second time, but essentially the same results occurred. It is, therefore, believed that non-specific factors are involved.

Table 22

B. rodhaini Challenge 59 days after Vaccination

Vaccine	No. Reacting	Avg. High Parasitemia	Deaths	Time of Deaths
BR #1	1/5	0.6	0/5	-
BR #2	4/4	3.5	0/4	-
BR #3	4/4	19.0	1/4	15 da.
BR #4	4/4	19.4	1/4	15 da.
BRV #1	4/4	1.6	0/4	-
BRV #2	5/5	4.9	0/5	-
BRV #3	4/4	60.2	4/4	15 da.
BRV #4	5/5	20.6	1/5	8 da.
VB Control	4/4	13.2	0/4	-
Control	5/5	52.8	5/5	9 da.
		$P<0.01$		
	DRS:	36		

Table 23

B. rodhaini Challenge 101 Days After Vaccination

Vaccine	No. Reacting	Avg. High Parasitemia	Deaths	Time of Death
BR #1	1/3	0.07	0/3	-
BR #2	3/4	32.0	0/4	-
BR #3	5/5	26.9	2/5	12 da.
BR #4	3/4	8.4	1/4	12 da.
BRV #1	3/4	6.5	0/4	-
BRV #2	2/5	9.4	0/5	-
BRV #3	4/5	31.2	1/5	15 da.
BRV #4	3/4	37.5	0/5	-
VB Control	5/5	17.8	1/5	12 da.
Control	5/5	84.4	5/5	10.4 da.
Significance		$P<0.01$		

DRS: 51

Toxicity Studies in Mice

4A65 and Ganaseg

Toxic reactions have been observed with 4A65, and in one instance a deer (D-159) receiving 4 mg/kg intravenously died approximately 10 minutes after injection notwithstanding efforts to revive the animal. No toxic signs have been observed with ganaseg, but it was decided to include this drug in order to better understand the possible tolerance levels associated with it.

Each drug was prepared so that the following drug levels could be administered I.P in 0.5 ml: 2-4-8-16-32-64-128 and 256 mg/kg. A total of 80 mice were used. Five mice were injected at each dose level for each of the drugs used. LD 50's were determined by the Reed-Muench method. All deaths occurred within 24 hours, but animals were observed for a 6 day period after the administration of the drug.

The LD 50 on ganaseg was 89.2 mg/kg. The LD 50 on 4A65 was 49.3 mg/kg. The tolerance of these drugs was higher than expected, but it is probable that the route of administration is significant. Previous toxic signs in cattle and deer are more severe when the drug is given I.V.

Fractionation and Testing of *Theileria cervi* CF Antigens

A better understanding of the serologic and immunologic relationship between various *Theileria* organisms will undoubtedly become an important factor in future control programs in Africa and elsewhere. The only *Theileria* available for study in the U.S. is *T. cervi*. Using this organism as a model, limited trials have been undertaken to better characterize the antigens present. Complement-fixation antigens can be readily prepared, as shown in past publications, but antigen titrations of various fractions and preparations to better locate the greatest concentrations have not been made.

The methods of quantitating antigens and antigen fractions for *Anaplasma* have been used to accomplish the same with *Theileria*.

Antigen Production:

Splenectomized deer 161 was used as a source of infected RBCs when the *Theileria* parasitemia reached 20%, with a 24% PCV. A total of 400 ml blood was collected in sodium citrate. The RBCs were washed 4 times in veronal buffer (VB) pH 7.2, and packed by centrifugation at 650 x G for 15 minutes. A total of 45 ml packed RBCs were lysed in 10 volumes of distilled water. Antigens A-1, A-2, and A-3 were prepared from these water lysed cells. A second 45 ml aliquot of packed RBCs were lysed in 10 volumes of distilled water saturated with CO_2. Antigen B was prepared from cells lysed in CO_2 water. All lysed cells were allowed to stand overnight at 5°C.

Water lysed cells were centrifuged 40 minutes at 35,000 x G. The supernate was discarded, and the sediment was mixed with distilled water and centrifuged, a second time, 40 minutes at 35,000 x G. The supernate was removed by suction. A light weight sediment was then poured off. This sediment was largely fluid and weighed 10.03 gms. To this, 40 ml VB was added giving a 20% suspension and then mixed in a Vir-tis tissue homogenizer at full speed. This preparation was labeled Antigen A-1.

Following the second centrifugation and after pouring off the sediment making up Antigen A-1, a second sediment was removed. This sediment, while heavier than A-1, still appeared to be a light weight poorly packed sediment. This was removed by carefully teasing the sediment off with a spatula. This material weighed 9.0 grams. A 20% suspension in VB was prepared, mixed in the Vir-tis homogenizer, and labeled Antigen A-2.

A dark, densely packed sediment remained after removing the 2 previous ones (A-1 and A-2). This material weighed 0.5 grams. A 1% suspension in VB was prepared, and the sediment suspended by sonication. This antigen was labeled A-3.

Antigen B was prepared from the sediment collected in the CO_2-H_2O lysed material. The lysed cells were centrifuged 40 minutes at 35,000 x G, and the sediment saved. This sediment was washed 2 times with CO_2 water, with centrifugation each time being 40 minutes at 35,000 x G. The final sediment weighed 4.34 gms. An 8% suspension was prepared using 49.9 ml VB, homogenizing by sonication 4 minutes at 8 watts.

All antigens, A-1, A-2, A-3 and B, were frozen the day of preparation at-20°C. They were later thawed, sonicated lightly, and then tested using

a protocol similar to that employed in the anaplasmosis CF tests. Antigen end points were considered the amount of antigen capable of giving complete fixation of complement in the presence of a known positive Theileria sera and 2 units of complement. The hemolytic system was the same as used for anaplasmosis tests; 2 units of amboceptor plus 2% sheep red cells.

All antigens were tested at 1:4 dilutions in 0.05, 0.1, 0.2, 0.3, and 0.4 amounts to establish end points. Where 4+ fixation failed to occur, a regression analysis was calculated, and the end point estimated on the basis of regression coefficient (b).

Results:

The unit titer, considered the number of ml of undiluted antigen capable of complete fixation, the total number of units present in each antigen, and the concentration expressed as units per gram of sediment are recorded in Table 24. The very light supernate (A-1) was, as expected, the least active of all antigens tested. No end point was reached in the titration used, so the end point was projected mathematically as indicated. The A-2 or intermediate sediment was moderately active with a total of 600 units having been recovered. The concentration of this antigen, while 2.3 times that of A-1, was low in comparison to other antigens.

The heavy sediment (A-3) contained most of the antigen with 1333 total units having been recovered. The weight of this sediment was very low, hence, the concentration reached 2,667 U./gm. which is approximately 93 times greater than A-1, and 40 times greater than A-2.

The B. antigen, or that material prepared from CO_2 saturated water, showed moderate response, with a total unit number being 665. Anaplasma antigens generally showed greater relative activity than this. In the past, CO_2 antigens have been almost equal to the total antigenic activity of the various water lysed fractions. In this instance a total of 2,221 units were recovered from the 3 water lysed antigens, which is 3 times more than was seen with the CO_2 antigen. The reason for this is unknown, but there does appear to be some evidence of specific denaturation or antigen loss associated with the CO_2 treatment.

Table 24

Comparative Activity of *Theileria* Antigens

Antigen	1:1 dil. Unit Titer	Total No. of Units	Units/ gm. of Sediment
A-1	0.694 ml	288	29
A-2	0.075 ml	600	67
A-3	0.0375 ml	1333	2667
B	0.075 ml	665	153

A-1: Water lysed antigen lightest sediment.

A-2: Water lysed antigen light sediment.

A-3: Water lysed antigen heavy sediment.

B: CO_2 water lysed antigen.

Trypanosoma theileri

Transmission Attempts and Serial Transfer on Artificial Media

(In cooperation with Dr. T.J. Galvin)

Trypanosoma theileri was frequently encountered in Colombia in those areas where other blood parasites were common. This organism was not, however, commonly encountered in the Bogota area at altitudes of 8,000 feet or over. We have repeatedly found *T. theileri* infections in cattle maintained on the Research farm of the Veterinary School at College Station, Texas.

This organism is generally considered non-pathogenic, however, critical studies of its pathogenicity do suggest some pathologic changes under some circumstances, particularily in splenectomized calves. It is not considered a pathogen of economic importance, unless it may have some influence on other infections. Our reason for studying this organism is its resemblence to other pathogenic, bovine trypanosomes, the ease in which it is cultivated on artificial media, and our curiosity concerning the possible relationship to other pathogens.

There are 2 cows maintained in the service herd, which when cultured, are consistently positive to *T. theileri*. Cultures are routinely made by inoculating 0.5 to 1.0 ml of the blood to be checked on to blood agar slants, and incubated 28° - 30°C. for several days. The blood agar is prepared to contain 200 U. of penicillin, and .25 mg streptomycin per ml to retard bacterial growth. Cultures are checked by making wet mounts every 2 days for at least 8 days. In positive samples, growth is often seen at 2 days, and nearly always at 4 days.

A preliminary transmission trial was made using 8 ml of fluid harvested from a blood agar culture that was heavily infected with T. theileri. This material was injected I.V. into splenectomized calf 206, that was negative on culture for T. theileri. Blood from Calf 206 was cultured on blood agar slants the day of injection and on days 1, 2, 5, 8, 12, 15, 21, and 42 for evidence of T. theileri infection. Calf 206 remained negative for the entire time.

A second attempt at transmission consisted of inoculating I.V. 10 ml of whole blood from a carrier cow into a splenectomized calf. Cow 673, a known carrier positive to blood agar culture, was the source of infective inoculum for splenectomized calf 142. Cow 693, a known carrier positive to blood agar culture, was the source of infective inoculum for splenectomized Calf 426. After exposure calves 142 and and 426 were cultured at frequent intervals for the detection of T. theileri. In addition, PCV and parasitemia were conducted at regular intervals for evidence of change associated with infection.

Results of culture and PCV are recorded on Table 25. No trypanosome parasitemias were observed in either calf on stained smears. Evidence of infection and successful transmission was observed in both calves. Calf 142 showed evidence of a circulating T. theileri parasitemia on days 4 and 5. No recurring parasitemias were detected in calf 142. Calf 426 showed evidence of a trypanosome parasitemia 6 hours after inoculation. This more likely reflects trypanosomes inoculated. Positive cultures were also observed on days 3, 4, 5, and 6. A recurring parasitemia was detected on culture on days 22, 25, 29, and 32. Calf 426 remained negative thereafter.

There was no evidence that trypanosome infection was associated with changes in PCV.

Calf 142 developed evidence of eperythrozoon at about the same time C-426 was showing a positive trypanosome parasitemia; whether or not the eperythrozoon was in any way responsible for the absence of T. theileri is unknown.

Conclusions:

Even though evidence was affirmative that Trypanosoma theileri was passaged in calves, the animal response to infection was minimal and as far as could be determined was not persistent. Cultures will be made 275 days after exposure, but this has not yet been accomplished.

Table 25

Transmission of *Trypanosoma theileri* to Splenectomized Calves

Time of Culture	Calf 142 Culture Results	Calf 142 PCV	Calf 426 Culture Results	Calf 426 PCV
0	-	28	-	25
6 hours	-		+	
24 hours	-		-	
30 hours	-		-	
2 days	-		-	
3 days	-		+	
4 days	+	31	+	29
5 days	+		+	
6	-		+	
7	-	29	-	26
8	-		-	
11	-	29	-	26
13	-	29	-	20
15	-	29 Epy.*	-	24
20	-	28 Epy	-	23
22	-	26	+	23
25	-	22	+	22
29	-	18	+	22
32	-	16	+	22
39	-	20 Epy.	-	22
48	-	22 Epy.	-	23
56	-	17 Epy.	-	24
70	-	19	-	23
77	-	22	-	23
84	-	23	-	24
100	-	23	-	22

Epy.: Eperythrozoon

Infectivity and Immunogenicity of Irradiated *Babesia rodhaini*

J. P. Bishop and K. L. Kuttler

Babesia rodhaini is a protozoan parasite of the red blood corpuscles of rodents first isolated from *Thamnomys surdaster surdaster* in the Congo in 1950 (21). *B. rodhaini* was easily transmissible to laboratory mice (21) and became increasingly virulent with repeated serial transfers in mice (6). Colas-Belcour and Vervent (6) found that the majority of mice died from the infection, but those which recovered were resistant to challenge.

B. rodhaini in rodents has been used widely as a model for the study of *Babesia* infections of cattle, especially in the field of chemotherapy. The immunology of *B. rodhaini* in rats and mice has recently received attention (9,11,12,17,18,20). Attempts to induce protective immunity in rats and mice by use of irradiated *Plasmodium berghei* have been relatively successful (1,7,8,13,14,15,22,23,24,25). Recent studies (19) indicated that essentially similar results could be achieved in mice and rats by irradiation of the erythrocytic forms of *B. rodhaini*.

The present series of investigations was undertaken to further study the effect of various gamma radiation dosages on the infectivity and immunogenicity of erythrocytic stages of *B. rodhaini*.

Materials and Methods:

The strain of *B. rodhaini* used throughout this study was obtained from Dr. C. G. Ludford at the Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan, and was maintained by serial

passage in albino mice in the Institute of Tropical Veterinary Medicine, Texas A&M University, College Station, Texas.

Male inbred albino mice weighing 30-40 gm. each were used as experimental hosts. The organisms used for immunization and infection were prepared as follows: blood containing 60-70% parasitized erythrocytes was harvested from infected mice seven days after 0.5 ml intraperitoneal (ip) inoculation of a 1:1000 dilution of B. rodhaini parasitized mouse blood. The number of parasitized erythrocytes per ml. was determined by red blood cell counts and thin-blood smears stained with Giemsa stain.

The inoculum was exposed to the desired amount of irradiation using a 800 c source of cobalt-60. The 800 c source delievered a dose rate of approximately 1550 r/minute. The inoculum was kept in an ice bath until it was irradiated and afterwards until the mice were injected. Other inocula were maintained under similar conditions for the duration of irradiation and were used as nonirradiated controls. All inoculations and challenges were given ip.

The first series of experiments was undertaken to determine the effect of gamma radiation on the infectivity of B. rodhaini in mice. Seventy mice were separated into seven groups. The mice of the first group received 7×10^5 nonirradiated B. rodhaini parasitized erythrocytes. The other six groups received approximately the same number of parasitized erythrocytes irradiated respectively with 10,000 r, 14,000r , 18,000 r, 22,000 r, 26,000 r and 30,000 r. All mice were examined daily for parasitemia by the use of Giemsa stain on thin-blood smears.

In a second experiment the effect of gamma radiation on the immunogenicity of B. rodhaini was studied. Eight mice were inoculated with 7 x 10^5 parasitized erythrocytes irradiated at 30,000 r, and ten mice served as controls. All mice were examined daily for parasitemia by the use of Giemsa stain on thin-blood smears. All mice were challenged three weeks later with non-irradiated parasitized blood. Before challenge, blood from mice which had received irradiated parasitized blood was subinoculated into susceptible mice to determine whether a subpatent parasitemia might have resulted. Subinoculations were accomplished by diluting three drops of tail blood from each mouse with 0.5 ml of saline and injecting approximately 0.25 ml ip. into susceptible mice.

A third experiment was undertaken to evaluate the immunogenicity of parasitized and nonparasitized irradiated erythrocytes. Thirty mice were divided into two groups with one group receiving 7 x 10^6 parasitized erythrocytes exposed to 36,000 r and the other group receiving approximately the same number of nonparasitized erythrocytes exposed to 36,000 r. All mice were challenged three weeks later with nonirradiated parasitized blood and then examined daily for parasitemia by the use of Giemsa stain on the thin-blood smears.

Results:

The first series of experiments was undertaken to determine the effect of gamma radiation on the infectivity of Babesia rodhaini in mice. The results summarized in Table I show that all mice inoculated with nonirradiated B. rodhaini developed progressive infections and died seven to eleven days after inoculation. Mice infected with B. rodhaini parasitized erythrocytes

exposed to doses up to and including 22,000 r developed progressive parasitemias which were delayed in comparison to mice inoculated with nonirradiated B. rodhaini. A direct and significant ($p<0.01$) correlation between the irradiation dose and the time of death was noted. Some mice receiving parasitized erythrocytes irradiated at 26,000 r did not develop progressive infections. Infections were not detected in mice receiving parasitized erythrocytes irradiated at 30,000 r.

Based on the above results, a second experiment was undertaken to determine whether an inoculation with parasitized blood exposed to 30,000 r or higher stimulated a demonstrable resistance to a challenge infection with nonirradiated parasites. Before challenge, blood from the mice which had received irradiated parasitized blood was subinoculated into susceptible mice to determine whether a subpatent parasitemia might have resulted. No evidence of infection was discernible in these mice. After challenge with nonirradiated infected erythrocytes, the per cent survival was compared in the two groups of mice (Table II). The animals that were previously inoculated with irradiated parasitized blood all developed parasitemias and had a 50 per cent survival following the challenging infection. Although the mean survival time for the "immunized" mice was slightly longer than their controls, the difference between the two groups was not significant.

Since previous experiments (12) had indicated that resistance to B. rodhaini could be induced nonspecifically by substances other than of parasitic origin, a third experiment was designed to determine whether the irradiated parasites were responsible in stimulating the observed

acquired resistance. As indicated in Table III, the animals previously inoculated with irradiated parasitized blood all developed parasitemias and had a 25 per cent survival following the challenging infection, whereas the animals previously inoculated with irradiated nonparasitized blood had a zero per cent survival following the challenging infection. No significant difference was observed in the mean survival time among the animals in the two groups.

Conclusions:

The results obtained in studies designed to determine the minimum irradiation dosage which would inhibit multiplication of *Babesia rodhaini* indicate that progressive infections were prevented when the parasites were exposed to 30,000 r. Subinoculations into susceptible mice from parasites thus treated failed to produce active infections. These results confirm and extend those reported recently from cattle inoculated with irradiated *B. bigemina* (5) and from mice and rats inoculated with irradiated *B. rodhaini* (19).

An analysis of the foregoing experiments seems to indicate the development of acquired resistance to *B. rodhaini* in mice inoculated with irradiated *B. rodhaini*. The results of two separate experiments revealed that 25-50 per cent of mice inoculated once with irradiated *B. rodhaini* survived otherwise lethal infections. These results are in agreement with and extend those recently reported from mice and rats immunized with irradiated *B. rodhaini* (19). The results obtained in our studies compare favorably with the 10-30 per cent recovery rate among mice inoculated three to ten times with irradiated blood forms of *Plasmodium berghei* reported by Wellde and Sadun (24).

The injection of irradiated nonparasitized blood in the third experiment did not produce a discernable acquired resistance to B. rodhaini. Presumably the irradiated parasitized blood was responsible for the development of acquired resistance to B. rodhaini.

The observation that inoculation of mice with irradiated B. rodhaini may induce protective immunity, suggests that the presence of replicating Babesia in the host is not necessary for the development of acquired resistance. This is in agreement with the concept of "sterile immunity" described for B. argentina (4), B. bigemina (2,3), B. divergens (10), B. microti (9), and B. rodhaini (9,16).

Summary:

Babesia rodhaini parasitized mouse blood exposed to varied doses of gamma radiation up to 30,000 r was inoculated into mice. Mice inoculated with nonirradiated B. rodhaini developed progressive infections and died 7 to 11 days after inoculation. Mice infected with B. rodhaini parasitized blood exposed to doses up to and including 22,000 r developed progressive parasitemias which were delayed in comparison to mice inoculated with non-irradiated B. rodhaini. Some mice receiving parasitized blood irradiated at 26,000 r did not develop progressive parasitemias. Progressive infections were prevented by exposure to irradiation at 30,000 r.

The results of two separate experiments revealed that one inoculation of parasitized blood exposed to 30,000 r or higher apparently stimulated a resistance to a challenge infection with nonirradiated parasitized blood. While 20 out of 20 control mice died as a result of challenging infections, 9 our of 28 mice previously exposed to irradiated parastitized blood survived.

The injection of irradiated nonparasitized blood did not produce a discernable acquired resistance to B. rodhaini. Presumably the irradiated parasitized blood was responsible for the development of acquired resistance to B. rodhaini.

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Table I

Infectivity of Irradiated *Babesia rodhaini* Parasitized Mouse Blood

Radiation dosage in roentgens[a]	No. with parasitemia/ No. inoculated[b]
0,000	10/10
10,000	10/10
14,000	10/10
18,000	10/10
22,000	10/10
26,000	8/10
30,000	0/10

a size of inoculum: 7×10^5 parasitized red blood cells.

b detected by either thin-blood films or subinoculations.

Table II

Immune Response of Mice Inoculated Once with

Irradiated *Babesia rodhaini*

Treatment	No. Survivors/No. Challenged
	(Challenge Dose--$7x10^4$ parasitized RBC's)
$7x10^5$ parasitized RBC's exposed to 30,000	4/8
No treatment	0/10

Table III

Immune Response of Mice Inoculated Once with Irradiated Parasitized and Nonparasitized Blood

Treatment	No. Survivors/No. Challenged
	(Challenge Dose--$7x10^2$ parasitized RBC's)
$7x10^6$ parasitized RBC's exposed to 36,000 r	5/20
$7x10^6$ normal RBC's exposed to 36,000 r	0/10

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Publications

Publications (1970)

1. 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) p. 879-885.

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all repeated from '69 list

Manuscripts (Submitted or accepted for publication)

1. Kuttler, K.L., Graham, O.H., and Johnson, S.R.: Boophilus annulatus as a Vector for Anaplasmosis in Cattle and White-tailed Deer (Odocoileus virginianus) (Accepted for publication in the Journ. of Paras.)

2. Corry, G.L., Kuttler, K.L.: Serological Activity of a Soluble Antigen of Theileria cervi., Submitted to "Experimental Parasitology"

repeated '73 list

3. Bishop, J.P. and Kuttler, K.L.: Infectivity and Immunogenicity of Irradiated Babesia rodhaini. Submitted to "The Journal of Protozoology" repeated in '73 list

4. Zaraza, H., Kuttler, K.L.: Comparative Efficacy of Different Immunization Systems Against Anaplasmosis. Accepted for publication in "Tropical Animal Health and Production" 11 Dec., 1970.

5. Kuttler, K.L.: Therapeutic Efficacy of Oxytetracycline and a Dithiosemicarbazone in the treatment of anaplasmosis. Accepted for publication in Am. J. Vet. Res.

Future Work

Anaplasmosis.

1.) Therapeutic studies will continue, being directed primarily at the removal of the carrier status, using the following drugs:

   a.) Oxytetracycline

   b.) 356C61 } based on availability

   c.) 4A65 }

2.) Methods for prevention of anaplasmosis, by means of a killed or live vaccine will continue.

   a.) Detection of *Anaplasma* antigens in tissues other than blood.

   b.) Purification and/or concentration of *Anaplasma* antigens.

   c.) Survey of available adjuvants.

   d.) Actual vaccine trials should be initiated during the coming year.

3.) Field tests for the control of anaplasmosis in Texas based on a test and treatment program is planned if drugs become available.

4.) Duration of immunity trials using the Diamond Vaccine will continue.

Babesiosis:

1.) Attempts will be made to adapt *Babesia rodhaini* to rabbits, and guinea pigs.

2.) Further vaccination studies will be made, modifying the route of inoculation in mice.

3.) Culture will be made. Efforts at growth of the causitive organism on tissue.

Theileria:

1.) Based on the availability of deer, further studies will be conducted on serologic aspects of theileriosis and the possible immunologic and serologic relationship with anaplasmosis.

2.) Attempts to grow *T. cervi* on tissue culture are planned.