AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D. C. 20523

BIBLIOGRAPHIC INPUT SHEET

FOR AID USE ONLY
0170

1. SUBJECT CLASSIFICATION

A. PRIMARY
Agriculture AL72-0000-0000

B. SECONDARY
Pests of animals

2. TITLE AND SUBTITLE
Immune response of cattle inoculated with irradiated Babesia bigemina

3. AUTHOR(S)
Bishop,J.P.

4. DOCUMENT DATE
1971

5. NUMBER OF PAGES
151p.

6. ARC NUMBER
ARC

7. REFERENCE ORGANIZATION NAME AND ADDRESS
Tex.A&M

8. SUPPLEMENTARY NOTES *(Sponsoring Organization, Publishers, Availability)*
(Diss.-Tex.A&M)

9. ABSTRACT

10. CONTROL NUMBER
PN-RAA- 119

11. PRICE OF DOCUMENT

12. DESCRIPTORS
Babesia
Complement fixation
Immunity
Radiation

13. PROJECT NUMBER

14. CONTRACT NUMBER
CSD-1947 Res.

15. TYPE OF DOCUMENT

AID 590-1 (4-74)

IMMUNE RESPONSE OF CATTLE INOCULATED WITH IRRADIATED *BABESIA BIGEMINA*

A Dissertation

by

JOHN PHILIP BISHOP

Submitted to the Graduate College of
Texas A&M University in
partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

December 1971

Major Subject: Veterinary Microbiology

IMMUNE RESPONSE OF CATTLE INOCULATED WITH

IRRADIATED *BABESIA BIGEMINA*

A Dissertation

by

JOHN PHILIP BISHOP

Approved as to style and content by:

L. C. Grumbles
(Co-Chairman of Committee)

R. A. Todorovic
(Co-Chairman of Committee)

L. C. Grumbles
(Head of Department)

L. G. Adams
(Member)

Phillip A. Lincoln
(Member)

Fred D. Mauer
(Member)

December 1971

ABSTRACT

Immune Response of Cattle Inoculated with Irradiated *Babesia bigemina*

(December 1971)

John Philip Bishop, B.S., D.V.M., M.S., The Ohio State University

Directed by: Drs. L. C. Grumbles and R. A. Todorovic

Experiments were performed to separate *Babesia bigemina* from *Babesia argentina*, *Babesia major*, and *Anaplasma marginale*. The method of separation was rapid passage through 5 splenectomized calves. Five blood passages were carried out in 6½ days. *Babesia argentina*, *B. major*, and *A. marginale* were eliminated as contaminants after 4 passages. A frozen stabilate of the isolated *B. bigemina* was established.

A method for the preparation and examination of combination thin and thick blood films for the detection of *Babesia* parasitemia was developed. The technique for the staining of the combination thin and thick films involved the use of a phosphate buffered Giemsa stain solution containing alkyl phenoxy polyethoxy ethanol. Babesial complement fixation (CF) antigens were also prepared and titrated for use in a CF microtiter procedure.

Babesia bigemina parasitized blood exposed to varied doses gamma radiation up to 60 kRad was inoculated into calves. Calves infected with 1×10^{10} *B. bigemina* parasitized erythrocytes exposed to doses up to and including 30 kRad developed progressive parasitemias. Some calves receiving 1×10^{10} parasitized erythrocytes

irradiated at levels of 36 and 42 kRad did not develop progressive infections. Progressive infections were prevented by exposure to irradiation at 48 kRad or higher. Subinoculations into susceptible splenectomized calves from parasites thus treated failed to produce active infections.

In addition to a lower infection rate brought about by irradiated parasites, calves that did become infected had prolonged prepatent periods and lower maximum parasitemias. Control calves, however, that had been inoculated with 1×10^7 nonirradiated parasitized erythrocytes also had prolonged prepatent periods and lower maximum parasitemias. Therefore, the prolonged prepatent periods and lower maximum parasitemias in calves that had received 1×10^{10} irradiated B. bigemina could have been partly due to a reduction in the number of viable parasites injected. The finding that calves inoculated with 1×10^7 nonirradiated parasitized erythrocytes had prolonged prepatent periods and lower maximum parasitemias indicate that such a standardized inoculum might be of use as a vaccine to produce an attenuated infection.

A degree of acquired resistance to infection with B. bigemina developed in calves after 1 inoculation with B. bigemina parasitized blood irradiated at 48 and 60 kRad. The resistance was sufficient to suppress multiplication of the Babesia and to permit calves to survive otherwise severe clinical infections with nonirradiated parasites. There was also less erythrocytic destruction and a smaller increase in rectal temperatures following challenge.

Presumably, the irradiated parasites were responsible for the development of resistance since irradiated nonparasitized blood did not produce a discernable acquired resistance. The observation that inoculation of calves with irradiated B. bigemina stimulated a degree of protective immunity, suggests that the presence of replicating Babesia in the host is not necessary for the development of acquired resistance.

The acquired resistance to infection with B. bigemina developed in calves inoculated with 1×10^{10} B. bigemina irradiated at 48 and 60 kRad was similar to the acquired resistance developed in calves inoculated with 1×10^{10} nonirradiated B. bigemina. It seems likely that the protective immunity produced with irradiated B. bigemina may be similar to that produced with living pathogenic B. bigemina in non-fatal infections. The acquired resistance to infection with B. bigemina developed in calves inoculated with 1×10^{10} B. bigemina irradiated at 48 and 60 kRad was much greater than the acquired resistance to infection developed in calves inoculated with 1×10^{10} heat killed B. bigemina. Thus, it seems likely that immunization with irradiated Babesia may provide the special immunological properties of living parasites important for producing a strong immunity while suppressing the pathogenic effects of the parasite. The Babesia parasites could be irradiated and frozen without apparent loss of immunizing properties.

Dedicated to my wife

KAY

for her devotion, assistance, understanding, and encouragement

ACKNOWLEDGMENTS

To Drs. L. C. Grumbles, R. A. Todorovic, L. G. Adams, F. D. Maurer, and P. A. Linerode for suggestions, counsel, and interest during the course of this investigation and preparation of this manuscript;

To Dr. Alvaro Ariza, Radiofisico, for valuable technical assistance and the Instituto Nacional de Cancerologia, Bogota, Colombia for providing the irradiation facilities:

To Drs. D. E. Corrier, K. C. Thompson, and O. G. Vizcaino, fellow graduate students, for cooperation and assistance in collecting data for this study;

To personnel of the Instituto Colombiano Agropecuario and particularly to Drs. Guillermo Mateus and Gustavo Riveros for cooperation and assistance given to complete this investigation; to Mr. Luis Ramirez, Miss Nubia Carvajal, Miss Lidia Hincapie, Miss Nelly Giron, Mr. Jose Leal, and Mr. Luis Garcia for technical assistance;

To personnel of the Department of Veterinary Microbiology and the Institute of Tropical Veterinary Medicine at Texas A&M University and the Centro Internacional de Agricultura Tropical in Colombia for cooperation and assistance given to complete a fellowship financed by the Rockefeller Foundation and the United States Agency for International Development;

I wish to express my sincere gratitude.

TABLE OF CONTENTS

	Page
ABSTRACT	iii
DEDICATION	vi
ACKNOWLEDGMENTS	vii
TABLE OF CONTENTS	viii
LIST OF TABLES	x
LIST OF FIGURES	xi
INTRODUCTION	1
LITERATURE REVIEW	4
Immune Response of Cattle Inoculated with *Babesia*	4
Coinfectious Immunity	4
Development of Vaccination Technique	4
Difficulties Inherent in Vaccination Technique	6
Virulence of the Parasite in the Inoculum	6
Number of Infective Parasites Injected	10
Strain Immunity	11
Sterile Immunity	14
Detection of Babesial Complement Fixing Antibodies	14
Immunization with Irradiated Protozoa	19
Background Information	19
Effects of Radiation on Protozoa	20
Immunization with Irradiated *Plasmodium*	23
Plasmodium gallinaceum	23
Plasmodium berghei	23
Plasmodium falciparum	31
Low Temperature Preservation of *Babesia*	31
MATERIALS AND METHODS	36
Experimental Animals	36
Experimental Organisms	37
Preparation of Irradiated and Challenge Inocula	40
Experimental Procedures	41
Assessment of Reactions	45
Parasitemia	45
Combination thin and thick blood films	45
Subinoculations	47

TABLE OF CONTENTS (continued)

Page

Packed Cell Volume..... 47
Rectal Temperature and Body Weights..... 48
Babesial Complement Fixing Antibody Levels..... 48
General..... 48
Hemolysin Titration..... 48
Complement Titration..... 49
Preparation of Antigen..... 50
Antigen Titration..... 51
Complement Fixation Microtiter Procedure..... 52

RESULTS..... 54

Separation of B. bigemina from B. argentina, B. major, and A. marginale..... 54
Experiment I..... 57
Experiments II and III..... 73
Experiments IV and V..... 88

DISCUSSION..... 116

SUMMARY..... 123

BIBLIOGRAPHY..... 126

APPENDIX..... 134

VITA..... 136

LIST OF TABLES

Table | Page

1. The Separation of B. bigemina from B. argentina, B. major, and A. marginale by Rapid Passage through 5 Splenectomized Calves..... 55

2. Characteristics of B. bigemina Infections Produced with Infected Blood Subjected to Different Radiation Doses. Summary of Experiments I, II, III, and IV..... 71

3. Immune Response of Calves Inoculated Once with Irradiated B. bigemina and Challenged 4 Weeks Later with Nonirradiated B. bigemina. Summary of Experiments IV and V..... 107

Appendix Table

A1. Composition of the Concentrates and Minerals Fed Experimental Animals..... 135

LIST OF FIGURES

Figure		Page
1.	*Babesia bigemina* parasitized erythrocytes on thin blood film. Giemsa. x1330	58
2.	*Babesia bigemina* parasites on thick blood film. Giemsa. x1330	59
3.	*Babesia bigemina* used for the preparation of antigens for the CF microtiter procedure on thick film. Giemsa. x1330	60
4.	Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 2 calves inoculated (I) with 1 x 10^9 *B. bigemina* parasitized erythrocytes exposed to 22 kRad and challenged (C) 7 weeks later with 1 x 10^{10} *B. bigemina* parasitized erythrocytes	62
5.	Complement fixation titers, % parasitized erythrocytes, % packed cell volume, morning rectal temperatures, and average daily gains for 2 calves inoculated (I) with 1 x 10^9 *B. bigemina* parasitized erythrocytes exposed to 26 kRad and challenged (C) 7 weeks later with 1 x 10^{10} *B. bigemina* parasitized erythrocytes	64
6.	Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 2 calves inoculated (I) with 1 x 10^9 *B. bigemina* parasitized erythrocytes exposed to 30 kRad and challenged (C) 7 weeks later with 1 x 10^{10} *B. bigemina* parasitized erythrocytes	66
7.	Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 1 calf inoculated (I) with 1 x 10^9 nonirradiated *B. bigemina* parasitized erythrocytes and challenged (C) 7 weeks later with 1 x 10^{10} *B. bigemina* parasitized erythrocytes	68

LIST OF FIGURES (continued)

Figure Page

8. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes morning rectal temperatures, and average daily gains for 1 calf inoculated (I) with 12 ml of normal blood exposed to 30 kRad and challenged (C) 7 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes..... 70

9. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 2 calves inoculated (I) twice with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 36 kRad and challenged (C) 3 weeks later after the last inoculation with 1 x 10^{10} B. bigemina parasitezed erythrocytes..... 75

10. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 2 calves inoculated (I) twice with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 42 kRad and challenged (C) 3 weeks after the last inoculation with 1 x 10^{10} B. bigemina parasitized erythrocytes..... 77

11. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 1 calf inoculated (I) with 1 x 10^9 nonirradiated B. bigemina parasitized erythrocytes and challenged (C) 5 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes..... 79

12. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 1 calf inoculated (I) twice with 12 ml normal blood irradiated with 42 kRad and challenged (C) 3 weeks after the last inoculation with 1 x 10^{10} B. bigemina parasitized erythrocytes..... 81

LIST OF FIGURES (continued)

Figure Page

13. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 2 calves inoculated (I) with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 36 kRad and challenged (C) 3 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes..... 84

14. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 2 calves inoculated (I) with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 42 kRad and challenged (C) 3 weeks after the last inoculation with 1 x 10^{10} B. bigemina parasitized erythrocytes..... 86

15. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 4 calves inoculated (I) with 1 x 10^{10} B. bigemina parasitized erythrocytes exposed to 24 kRad and challenged 4 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes..... 90

16. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 4 calves inoculated (I) with 1 x 10^{10} B. bigemina parasitized erythrocytes exposed to 36 kRad and challenged (C) 4 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes..... 92

17. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 4 calves inoculated (I) with 1 x 10^{10} B. bigemina parasitized erythrocytes exposed to 48 kRad and challenged (C) 4 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes..... 94

LIST OF FIGURES (continued)

Figure		Page
18.	Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 4 calves inoculated (I) with 1 x 10^{10} B. bigemina parasitized erythrocytes exposed to 60 kRad and challenged (C) 4 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes	96
19.	Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 3 calves inoculated (I) with 1 x 10^{10} nonirradiated B. bigemina parasitized erythrocytes and challenged (C) 4 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes	98
20.	Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 3 calves inoculated (I) with 1 x 10^{7} nonirradiated B. bigemina parasitized erythrocytes and challenged (C) 4 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes	100
21.	Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 3 calves inoculated (I) with 1 x 10^{4} nonirradiated B. bigemina parasitized erythrocytes and challenged (C) 4 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes	102
22.	Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 3 calves inoculated (I) with 1 x 10^{10} heat inactivated B. bigemina parasitized erythrocytes and challenged (C) 4 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes	104

LIST OF FIGURES (continued)

Figure Page

23. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 3 calves inoculated (I) with 10 ml normal blood irradiated with 36 kRad and challenged (C) 4 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes..... 106

24. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 4 calves inoculated (I) with 1 x 10^{10} B. bigemina parasitized erythrocytes exposed to 60 kRad and frozen, and challenged (C) 4 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes..... 110

INTRODUCTION

Parasitic protozoan induced immunities demonstrate many features in common with other microbiological infections but, because of the complicated life-cycles of protozoa, the phenomenon is not a simple reaction of the host to a single stage of the parasite. Instead, a variety of responses is evoked by forms which develop in the invertebrate host, by the final products circulating in the blood, and in some instances, by the continually changing antigenic pattern. Apart from the serum antibody response, recent work has indicated that cell-mediated immunity may be important in the host's defenses against certain protozoan parasites (Targett, 1968). The inconclusive results obtained with immunization using killed protozoa or protozoan fractions have been attributed to the complex and highly variable antigenic structures of the organisms and the loss or denaturation of the antigens during the preparation of the vaccines. Furthermore, the parasites may have been too rapidly destroyed in the host to elicit a discernible protective effect.

Although immunization against protozoan diseases has been studied in great detail, there has been little progress in the development of reliable vaccines. In fact, immunization has been

---

The citations on the following pages follow the style of Research in Veterinary Science.

put to practical use only against bovine babesiosis, bovine theileriasis, avian coccidiosis, and cutaneous leishmaniasis of man. In these instances, inoculation or exposure of living parasitic protozoa produces infections that result in protection of the host which unfortunately ensures continued transmission of the disease. The narrow margin between the induction of fatal disease and mild infections, resulting in protection, has led to the search for avirulent organisms that retain their immunogenic properties.

Irradiation of protozoa interferes with their physiologic processes and frequently inhibits their normal development and multiplication (Giese, 1967). Studies on the effects of ionizing radiations on parasitic protozoa have repeatedly shown that while an extremely high radiation dose is necessary to cause immediate death of the parasite, much lower doses can interfere with infectivity (Kimball, 1955). The immunogenicity of irradiated Babesia rodhaini (Phillips, 1970 & 1971b), Plasmodium spp. (Corradetti, et al., 1968; Nussenzweig, et al., 1969; Sadun, et al., 1969) and Trypanosoma spp. (Duxbury & Sadun, 1969 & 1970; Sanders & Wallace, 1966) in experimental animals (mice, rats, and owl monkeys) has been investigated. These studies have shown that parasites irradiated with a dose which abolished their reproductive potential and ability to produce patent infections may retain their capacity to produce an immune response in a susceptible experimental animal. Therefore, the use of irradiated parasitic protozoa as vaccines

might provide the special immunological properties of living protozoa while suppressing the pathogenic effects.

More information is needed from studies which evaluate the potential use of irradiated parasitic protozoa as vaccines against naturally occurring blood protozoan diseases where interactions among the host, parasite, vector, and environment are involved in promoting a state of stable immunity. Bovine babesiosis which occurs in the warmer areas of all continents offers a unique system to evaluate the use of irradiated vaccines against naturally occurring blood protozoan diseases. Bovine babesiosis is a naturally occurring tick-borne disease caused by species of Babesia which penetrate and destroy erythrocytes. The expert panel at the first Research Coordination Meeting of the joint Food and Agriculture Organization and International Atomic Energy Agency program on the use of isotopes and radiation in parasitology has recommended that radiation studies with Babesia be encouraged in view of their potential value in the development of vaccines (Anon., 1968).

The following series of investigations were undertaken to study the effect of various radiation dosages on the infectivity and immunogenicity of erythrocytic stages of Babesia bigemina. The effect of freezing on the immunogenicity of irradiated B. bigemina was also studied. The studies were conducted toward the ultimate goal of producing irradiated vaccines against bovine babesiosis.

LITERATURE REVIEW

Immune Response of Cattle Inoculated with Babesia

Coinfectious Immunity

Bovine babesiosis is an important tick-borne hemaprotozoan disease which occurs in the warmer areas of all continents and most major island groups. Cattle may be infected with *Babesia* by inoculation of blood containing living *Babesia* from either an acutely affected animal or a recovered carrier. Cattle infected in this way are relatively immune to natural infections. It is generally accepted that the immunity is based on premunition or coinfectious immunity (Sergent, *et al.*, 1924) in which the continued presence of relatively small numbers of organisms (often undetectable by routine methods) protects the host against additional overwhelming infections. Such immunization against bovine babesiosis has been practiced for many years in different parts of the world. In Queensland, Australia, vaccination against babesiosis has been commonly practiced on both fully and partially susceptible cattle in marginal tick infested areas for more than 70 years (Seddon, 1966).

Development of Vaccination Technique

The work of Pound, Hunt, and Tidswell (Tidswell, 1899 & 1900) in Australia led to a method of immunization against babesiosis.

Cattle recovered from the infection were used to provide infective blood to inoculate susceptible cattle. Until Legg's investigations of the 1930's showed Babesia argentina as well as B. bigemina to be present in Australia, only the latter organism was intentionally used in the vaccine (Legg, 1939). Following the isolation of strains of B. argentina, a vaccine incorporating both species was prepared (Anon., 1939).

Although blood from B. bigemina carriers did not always immunize, (Legg, 1931 & 1939; Tidswell, 1899 & 1900), these donors were favored because the reactions produced when "takes" were achieved were generally mild compared with those produced by blood taken during a primary parasitemia. Infective blood for vaccine was collected from steers that had organisms up to several months before. In North Africa, Sergent et al. (1945) emphasized that to achieve the desired degree of attenuation of B. bigemina, at least 3 months should have elapsed since the primary reactions of the donor.

The technique requires considerable technical control and specialist services. Some losses have occurred when very severe reactions have been provoked in inoculated cattle, and also when the natural disease has broken out in recently vaccinated cattle. The reaction of cattle to vaccination with Babesia is affected by at least 3 factors: the virulence of the parasites in the inoculum, the number of parasites injected, and the resistance of the individual.

Difficulties Inherent in Vaccination Technique

Virulence of the parasite in the inoculum. Since the inclusion of B. argentina in the vaccine in 1939 in Australia, Callow and Tammemagi (1967) reported that there has been uncertainty concerning results of vaccination with infective blood carrying this organism. Records of field inoculations indicated that vaccine supplied from carriers held at the laboratory produces a mild type of B. argentina reaction, and on occasion no observable reaction at all. Outbreaks of the natural disease within a few months of vaccination sometimes followed the use of infective blood.

Tsur (1961 & 1962) conducted vaccination trials against babesiosis using blood from latent and patent carriers. He, like Legg (1931 & 1939), found that blood from latent carriers of B. bigemina did not invariably infect susceptible cattle and did not provide a satisfactory immunity for a considerable proportion of inoculated animals. On the other hand, vaccination with blood from carriers having parasites in the peripheral circulation may cause violent reactions and may necessitate specific drug treatment.

Callow and Tammemagi (1967) reported on the infectivity and virulence of blood from cattle latently infected with B. argentina. Confining the observations to 1-6 months after primary infections (the period when carrier animals are often used for babesiosis

vaccine), 5 ml of blood (the standard dose) collected from 6 different donors failed to infect 12 of 42 susceptible cattle inoculated subcutaneously (s/c).

Callow and Tammemagi (1967) found that blood from cattle undergoing a primary reaction with B. argentina was highly infective. All 126 intact cattle inoculated with blood from 6 reacting donors became infected and 78 of the reactions were classified as severe. The severe reactions were readily controlled with drug treatment. The difference in virulence between blood from cattle latently infected and blood from cattle undergoing a primary reaction was strikingly demonstrated in experiments with splenectomized calves. Whereas 10 of 19 calves inoculated intravenously (i/v) with 500 ml of blood from carriers survived without treatment, an acute reaction necessitating treatment developed in calves inoculated with 0.5-20 ml of blood from cattle undergoing a primary reaction with B. argentina. The work showed that variability in the infectivity of the blood of carrier animals used to provide vaccine and severity of the infections produced in cattle that could not be handled conveniently for drug treatment were obstacles to effective vaccination against babesiosis.

The work of Callow and Tammemagi (1967) resulted in the production of a new vaccine (Callow & Mellors, 1966). Babesia bigemina has been omitted from vaccine routinely supplied, because epidemiological investigations have confirmed the observations of Legg (1939) that this organism rarely causes outbreaks of babesiosis.

In special circumstances, B. bigemina is supplied as a separate vaccine. Anaplasma centrale is occasionally added to the B. argentina vaccine for use in herds where there is some risk of anaplasmosis. Calves 2 to 8 weeks of age are purchased from an area where babesiosis does not occur and splenectomized before use. Each week, calves are inoculated i/v with approximately 1×10^9 B. argentina. Four or 5 days after inoculation, the parasitemia is usually 0.75-2.0% and the blood is collected for the vaccine. The number of parasites per ml of blood is determined with reasonable accuracy from an estimate of the percent parasitized erythrocytes and the erythrocyte count. Blood taken at this time is also used to inoculate the next calf in the series.

Previously, the vaccine in Queensland was not standardized with respect to the number of parasites per dose, the number in the carrier's blood being unknown. Judging by the variations in the infectivity, the number of parasites per dose fluctuated markedly. Investigations of the minimum infective dose showed that 1×10^5 organisms injected s/c were not always an infective dose (Callow & Mellors, 1966). Infectivity of the new vaccine is insured by incorporating 1×10^7 to 1×10^8 B. argentina per dose. The number of parasites per dose used in vaccine on the day after collection is 1×10^7 organisms. To compensate for loss of infectivity during storage, the number of parasites per dose is gradually increased as the week progresses until it is ten times that used on the day after collection. To provide a practical

vaccine dose, blood from an uninfected steer is used as a diluent so that the parasite dose is contained in 5 ml of blood. The new vaccine is collected once per week, kept chilled at 1-5°C and dispensed as required. Most inoculations are performed by lay persons, usually owners. They are advised that reactions will probably occur 7-14 days after inoculation, and that they should be prepared to treat any severely affected cattle with a babesiacide.

Callow and Mellors (1966) reported that, following the use of over 400,000 doses of the new vaccine in Queensland, losses from severe reactions were less than 1%. They compared this figure with that of 5%, which was often suggested by experienced cattlemen and veterinarians to be the average mortality rate in partly or non-immune groups of cattle inoculated with the vaccine previously available.

Dalgliesh (1968) reported field observations in Queensland on the clinical effects of the new vaccine in fully and partially susceptible groups of cattle. Over 90% of 295 cattle in 5 groups with no previous exposure to ticks and over 30% of 380 cattle in 3 groups previously exposed to ticks had rectal temperatures of 105°F or more 7-13 days after inoculation. This indicated high infectivity of the vaccine, and a reasonably predictable reaction period for inoculated cattle under field conditions. Of the 675 cattle, 30 showed anorexia and signs of depression following inoculation. These and others showing rectal temperatures of 105°F

or more were treated with quinuronium sulfate at approximately half the dose rate recommended. No deaths attributed to inoculation occurred. From observations on groups inoculated during natural babesiosis outbreaks, it was considered probable that vaccination prevented further losses from babesiosis.

Number of infective parasites injected. Kemron et al. (1964) and Pipano (1969) reported that the length of incubation period for B. bigemina was inversely related to the number of parasites in the inoculum. They further stated that in intact cattle the severity of the reaction was directly related to the number of parasites in the inoculum. Legg (1928) was of the opinion that the number of parasites injected does not affect the length of the incubation period nor the intensity of the reaction. He obtained similar results when he inoculated 2 groups of susceptible cattle with 5 and 50 ml of infective blood,respectively. However, as he does not detail the number of organisms in the inoculum, it is conceivable that the 5 ml dose was already sufficient to elicit a response customary in intact animals inoculated with blood containing B. bigemina.

Callow and Tammemagi (1967) reported that on occasions, blood from carriers with B. argentina was infective as a 100 ml dose (i/v), but not as a 5 ml dose (s/c), indicating that a minimum dose is required for infection at least by the s/c route. Failures in transmission occurred despite the fact that blood from carrier

animals was used immediately after collection. Had the same blood been dispatched for use in the field, fewer transmissions would probably have resulted because of decreases in live parasite numbers to levels below the minimum infective dose.

Callow and Mellors (1966) investigated the minimum infective dose for B. argentina and showed that 1×10^5 organisms injected s/c were not always an infective dose. Infectivity was ensured by incorporating 1×10^7 to 1×10^8 B. argentina per dose.

Strain immunity. It is known that immunity to B. bigemina following mechanical or tick infection in the laboratory is not always sufficient to prevent clinical reinfection when the animal is subjected to field exposure. Likewise, cattle moved from one area of enzootic B. bigemina to another may develop a clinical infection. It is generally accepted that these super-infections are due to strains of different antigenicity.

Summerville (1960) stated that cattle that have recovered from a babesial infection and have eliminated the parasite, may not react to reinfection from the same source, but may do so after exposure to a field strain. Mahoney (1962) in his survey of Babesia infections in cattle in an enzootic area found an almost continuous parasitemia in the blood between 6 months and 2 years of age, as opposed to the long periods of latency with occasional transient parasitic recrudescences which occurred in cattle after a single infection. Mahoney explained the continuous parasitemia by supposing that the presence of one infection does not greatly prejudice

the establishment of a second. In this way, a succession of separately induced parasitic waves might give rise to an intermittent or even continuous parasitemia depending on the number of super-infections received. Mahoney does not discuss the reasons why super-infections are possible, but if cattle can successfully suppress the parasitism of a single strain and not those of reinfecting strains, then the reinfecting strain may be antigenically different. It seems likely that even with a single infection, the parasitic relapses which occur are with antigenic variants of the original inoculum.

Phillips (1969) reported the isolation of a population of B. rodhaini which was resistant to antiserum in passive transfer tests. The production of an antiserum-resistant population would be brought about either by selection and multiplication of less susceptible parasites already present in the original population or by an antigenic variation in B. rodhaini in response to antiserum. Phillips (1971a) reported that B. rodhaini can undergo antigenic variation during the course of an infection.

Callow (1967) investigated the acquired immunity of cattle to B. bigemina by challenging splenectomized and intact cattle with either the original infecting strain or with one to which the cattle had not been previously exposed. The result of the challenge depended, in most instances, on which strain was used, and on whether or not challenged cattle were carriers. Results ranged from complete sterile immunity in homologously challenged,

self-cured cattle, through partial susceptibility with virtually asymptomatic parasitemias in heterologously challenged carrier cattle, to extreme susceptibility in heterologously challenged, self-cured cattle. The work showed that there was an immunological difference between the 2 strains and that cattle could possess a high degree of homologous immunity in the absence of heterologous immunity. It also demonstrated the relative nature of acquired immunity in babesiosis. Although parasitemias were produced in every case following heterologous challenge of carriers, these were less intense than those in controls, and generally were not accompanied by clinical symptoms. The degree of heterologous susceptibility depended on whether or not cattle were carriers. Parasitemias following challenge in carriers were much lower than in self-cured cattle. The latter either died or seemed likely to do so without treatment, whereas carriers had little difficulty in controlling their parasitemias.

Callow (1968) stated that homologous strain immunity may have a two-fold importance in the study and control of babesiosis. First, in evaluating the production of acquired immunity to natural infection by various vaccination procedures, challenges should be with a heterologous strain. Secondly, for the long term maintenance of immunity to babesiosis by regular re-vaccination, either some antigenic variation should be introduced into the vaccine strain, or the strain changed regularly to one capable of causing a mild parasitemia in previously vaccinated cattle.

Sterile Immunity

The classical concept of "premunition" or coinfectious immunity, propounded by the Algerian workers Sergent, et al. (1924) was derived largely from observations on bovine piroplasmosis including B. bigemina infections. A sterile immunity has been demonstrated with B. bigemina (Callow, 1964 & 1967), B. argentina (Callow, 1968), and Babesia divergens (Joyner & Davies, 1967) infections in cattle, with B. rodhaini infections in rats (Phillips, 1969) and B. rodhaini and Babesia microti infections in mice (Cox & Young, 1969).

Callow (1967) reported that homologous immunity in self-cured cattle developed quickly and was sufficient to prevent parasitemias in 4 cattle challenged 12-14 weeks after primary infection. The immunity did not appear to decrease as there was no increased tendency for cattle to show parasitemia even with long intervals between challenges. Homologous immunity in a number of instances was apparently sufficiently solid to prevent animals being reinfected. Yet it was shown that such an animal could have very little immunity to heterologous challenge even when this was performed only 2 weeks after a homologous challenge.

Detection of Babesial Complement Fixing Antibodies

Hirato et al. (1945) first reported the use of a complement fixation (CF) test to detect Babesia caballi in horses. The

antigen consisted of stromata of erythrocytes from horses acutely infected with B. caballi. These authors found that antibodies became detectable by the CF test 11 to 15 days after the onset of parasitemia and persisted for at least 100 days.

Mahoney (1962b) developed a CF test for the detection of antibodies to B. argentina and B. bigemina. The CF test was based on crude antigens prepared from infected erythrocytes by lysis with distilled water, followed by centrifugation. The antigen was shell-frozen in 1.0 ml quantities at -79°C and then preserved at -20°C. Homologous antibodies became detectable to the CF test within 7 to 21 days after infection and remained detectable during the acute and convalescent stages of infection. The antigens were apparently species-specific and reacted with heterologous serums at low titers.

Mahoney (1964) determined weekly levels of CF antibody for homologous antigen in calves subjected to experimental tick transmitted infection with either B. argentina or B. bigemina. With calves infected with B. argentina, 95-100% of serum samples contained antibody at or above the minimum diagnostic level for the ensuring 7 months, and after 5 more months, the proportion declined to 50%. In 9 calves infected with B. bigemina the proportion of positive serum was 94-100% during the first 4 months, declining thereafter to the low figure of 16% at 12 months. However, in all but 3 of these 20 cattle, the continued presence of parasites during periods of 12-18 months after infection was proved by the

subinoculation of 500 ml of blood into susceptible, splenectomized calves. Thus in these proven carriers, CF antibody tended to fall below the diagnostic level before infection was finally eliminated.

Schindler and Denning (1962) prepared a CF antigen from erythrocytes of dogs acutely infected with Babesia canis by means of immunohemolysis and, more recently, by exposing the erythrocytes to 0.3% saponin. The antibody became detectable to the CF test within 12 to 34 days following infection. No serological cross-reaction was observed between B. rodhaini and B. canis using the antigen of the latter parasite. Lyophilization was found to be an efficient means for long term preservation of the antigen. Schindler et al. (1966) inoculated dogs with live B. canis and soluble antigens from B. canis. Immunity was acquired only after infection with live B. canis. This resistance remained even after B. canis disappeared from the blood and even after disappearance of CF antibodies. Dogs inoculated with soluble antigens from B. canis produced CF antibodies but not protective immunity.

Mahoney (1967c) found that the most sensitive CF antigen for B. argentina was a crude suspension of the parasites prepared by distilled water lysis of infected erythrocytes followed by centrifugation. For B. bigemina, a specific, sensitive CF antigen was extracted from the parasite suspension with distilled water. In tests on serums obtained from cattle not exposed to B. bigemina, 1.6% reacted at a 1:5 dilution and 0.2% at a dilution of 1:10.

With cattle not exposed to B. argentina, 4.5% reacted at 1:5, 1.4% at 1.10, and 0.5% at 1:20. The nonspecific reactions to B. argentina were prevented by absorption of the serum with a suspension of normal bovine erythrocytic stroma. Cross-absorption tests indicated that the B. argentina and B. bigemina CF reactions each consisted of a major species-specific and a minor group-specific component.

Mahoney (1967a) found no positive correlation between the protective capacity and the CF titer of serum used in the passive immunization of calves against B. argentina. Mahoney (1967b) reported the application of the CF test in the evaluation of the immune response of cattle inoculated with killed B. argentina. The cattle reacted to the injections of killed B. argentina by the formation of CF antibodies, and subsequent challenge showed that a degree of immunity had been acquired as a result of the inoculations. Although the CF antibodies produced were probably not functional in immunity, their production indicated that effective stimulation by at least some of the babesial antigens had occurred. The CF antibody response during the course of infections with killed parasites was used to indicate the amount of antigenic stimulation that had occurred.

Frerichs et al. (1969) and Johnson et al. (1969) prepared antigens for the CF test for the evaluation of B. caballi and Babesia equi infections. Blood was collected at the height of parasitemia (3 to 7% for B. caballi; 60 to 85% for B. equi).

After centrifugation and removal of the supernatant plasma and buffy coat, the sedimented erythrocytes were lysed in 10 volumes of cold distilled water for 1 to 8 hours at 4°C. The lysate was passed through a refrigerated centrifuge. The parasites and erythrocytic stroma which collected in the form of paste on the wall of the centrifuge cylinder were mixed with veronal buffered saline, filtered and lyophilized for storage at -20°C. The serologic responses of 15 horses were studied by the CF test. The CF titer persisted longer in 14 of the horses than did their capability to transmit the disease, and thus was a reliable index of exposure to B. caballi.

Todorovic and Adams (1971) modified the preparation of B. argentina and B. bigemina suspensions and the extraction of CF antigens described by Mahoney (1967a). One volume of washed infected erythrocytes was resuspended in 10 volumes of veronal buffer and the erythrocytes were disrupted by sonification. After centrifugation, the supernatant fluid was collected and the procedure was repeated 3 times. The supernatant fluids were numbered serially in order of collection and were used as CF antigens. Specific antibodies were detected in the serum of cattle 8 days after blood-borne infection. A total of 5420 serum samples of cattle infected with babesiosis were tested. Approximately 95% of these samples were positive whereas about 5% gave discordant reactions.

Immunization with Irradiated Protozoa

Background Information

Many investigators have attempted to immunize against malarial infection by injecting hosts with non-living parasitic materials prepared by a variety of techniques, injected by several routes and accompanied by selected adjuvants. Zuckerman et al. (1967) have reviewed these attempts with mammalian malaria and concluded that in no case did such vaccination procedures provide "complete protection against infection with viable homologous parasites."

The inconclusive results obtained with killed vaccines have been attributed to the loss or denaturation of functional antigens during the preparation of the vaccines or to the rapid elimination of parasites in the host. Sinton (1939) postulated that the amount and rate of development of immunity to malaria may depend upon the amount and duration of antigenic stimulation. This suggestion was strengthened by the success reported by Weiss (1968) and Weiss and DeGuisti (1965 & 1966) in immunizing mice with a strain of Plasmodium berghei which had been rendered noninvasive by incubation in a tissue culture medium containing hamster serum. Thus, it has been postulated that antigenic substances associated with living, actively metabolizing parasites are important for producing a strong immunity.

Irradiation of the malarial parasite interferes with its physiologic processes and frequently inhibits its normal development and multiplication (Bennison & Coatney, 1945; Rigdon & Rudisell, 1945; Targett & Fulton, 1965; Ward, et al., 1960). The dose of irradiation needed to suppress multiplication appears to be considerably smaller than the lethal dose or that required to affect the plasmodia's metabolic processes. Ceithaml and Evans (1946) showed that erythrocytes parasitized by Plasmodium gallinaceum were capable of apparently normal oxygen uptake and glucose consumption after an X-ray dose of 10 kR which was sufficient to reduce the ability of the parasitized cells to increase in numbers after their intravenous injection into normal chickens. Therefore, irradiated parasites may retain the special immunological properties of living parasites while losing the pathogenic effects resulting from the reproductive capacity of the parasite involved.

Effects of Radiation on Protozoa

The nature of the ionizing radiation-killing event in bacteria and viruses is believed to be a radiation lesion in a molecule essential for replication. The probability of not being hit (or surviving) is given by $N/N_o\ e^{-IV}$, where N is the number surviving, N_o the original number present, N/N_o the survival ratio, e the base of the natural system of logarithms, I a constant, and V the sensitive volume. The size of the sensitive volume (the volume of

the molecule or molecules hit by the radiation) can be determined from plots of logarithms of the survival ratio against the dosage and is shown to be of the order of magnitude of a nucleoprotein molecule or a gene (Crowther, 1926; Hance & Clark, 1926; Setlow & Polland, 1962).

If the effect of radiation is expressed, in part, in metabolic changes, one might expect some alteration of the respiratory processes. Ceitham and Evans (1946) showed that erythrocytes parasitized by P. gallinaceum were capable of apparently normal oxygen uptake and glucose consumption after an X-ray dose sufficient to destroy reproductive capacity of the parasite (10 kR). Only when the dose was increased to 30 kR were these metabolic activities of the parasitized erythrocytes affected.

Ord and Danielli (1956) transferred nuclei from irradiated or nonirradiated Amoeba proteus to irradiated or nonirradiated enucleated cytoplasm, and found an LD_{50} of 290 kR was obtained from the cytoplasm. A dose, which will kill the protozoa if the nucleus is irradiated, has no apparent effect if the cytoplasm is irradiated. If the nucleus of a lethally radiated cell is replaced by a nonirradiated nucleus, the cell will recover, indicating that the cytoplasmic radiation damage is not lethal. If the irradiated nucleus is placed in a nonirradiated enucleated cytoplasm, it does not recover. It would seem that the nucleus is the site of greater and more permanent ionizing radiation damage.

To assess the nature of delayed killing of protozoa by ionizing radiation, it is necessary to study the rate of division for a prolonged period, perhaps for 30 or more generations. In such a study, Bridgman and Kimball (1954) showed that even after large doses Tillina magna and Colpoda sp. survive for several divisions before dying. The authors distinguish 4 types of death in Tillina: (1) death before the first postirradiation division; (2) death between the fourth and fourteenth postirradiation division; (3) death due to radiation but occurring at a constant rate during the life of the culture; and (4) death in non-irradiated controls. Of 198 clones of Colpoda exposed to dosages of 0-75 kR of X-rays, some soon recovered a normal division rate, others were markedly retarded but slowly recovered and were dividing at a normal rate by the end of the experiment. Still others continued to divide more and more slowly until they died. In general, the clones of Colpoda which eventually died out did not approach normal division rate even for one day, thus differing from Tillina, while lines which divided slowly for several days and then appeared to recover were fairly numerous in Colpoda, but not in Tillina. In general, delayed death is a complex phenomenon and appears to differ in various organisms.

The problem of suppression of fission is thought to involve several considerations: (1) damage to the cell division mechanism is gradually repaired during a series of divisions; (2) the extent of damage, measured by the degree of reduction of fission rate, is

a function of dosage; (3) rate of division is lowered when the number of fission-determiners is reduced below a required minimum; (4) recovery occurs only when the required minimal number of fission-determiners has been restored; (5) recovery is not always related to the number of postirradiation divisions; and (6) the rate of recovery of the cell is not related to the dose although absolute recovery at any time depends on the amount of damage sustained.

Immunization with Irradiated Plasmodium

Plasmodium gallinaceum. The immunizing capability of irradiated erythrocytic stages of malaria parasites was first reported by Ceithaml and Evans (1946). They found that chicken erythrocytes parasitized with P. gallinaceum exposed to 30 kR when injected into normal chickens conferred some degree of resistance to a challenge with normal parasites of the same strain.

Plasmodium berghei. Corradetti et al. (1966) reported 9 different experiments in which rats were inoculated with 3×10^7 erythrocytes parasitized with P. berghei irradiated respectively with 10, 14, 16, 18, 20, 22, 30, 30, and 40 kR. The rats of the first 4 groups, which were inoculated with parasites that had received from 10 to 18 kR, developed an infection which was delayed in comparison to that of the control rats. No variation in the severity of the infection and mortality was observed. On the contrary, the rats of the last 5 groups, inoculated with parasites

which had received an irradiation of 20 kR or more, did not develop any infection, while all the control rats became infected. The rats of the last 5 groups, 13-25 days after the inoculation with irradiated parasites, were submitted to a challenge of 4.5×10^5 erythrocytes containing nonirradiated parasites. The infection which followed was much shorter and lighter in comparison to that of the control rats: no mortality occurred in the previously inoculated rats, while mortality among the controls was 80%.

Corradetti et al. (1968) also reported an experiment in which mice were inoculated with the "Istisan" strain (passed from rat to rat for 20 years) of P. berghei irradiated with 30 kR. The mice were later challenged with the homologous strain and 75% of the mice showed resistance to the challenge, while all of the control mice became infected. These results were more favorable in comparison to those obtained in rats with the same strain in preceding experiments.

Wellde and Sadun (1967) reported results obtained from studies designed to determine the minimum irradiation dosages which would reduce significantly the survival and multiplication of Plasmodia. Rats infected with P. berghei parasitized erythrocytes exposed to doses up to and including 16 kR developed progressive parasitemias with obvious parasitic multiplication and reinvasion of erythrocytes. Some rats receiving parasitized erythrocytes irradiated at levels of 17 and 18 kR did not develop progressive infections. Infections were aborted by exposure to irradiation

at 19 kR or higher. Subinoculations from mice into susceptible mice which had received 19 kR irradiated parasites failed to produce active infections. The rats with aborted infections showed decreasing numbers of circulating parasites for 2 days and became negative on thick films usually by the third day following inoculation. The stained parasitic cytoplasm in these cells appeared pale blue and by the second day only nuclear material and pigment could be seen. Parasitic multiplication and reinvasion of erythrocytes were observed in all animals receiving inocula irradiated at 16 kR. However, patent parasitemias appeared considerably later and the number of parasitized erythrocytes increased at a much slower rate than in the controls receiving nonirradiated inocula. The minimum irradiation level (19 kR or more) which would reduce significantly the survival and multiplication of Plasmodia was essentially the same as that reported previously by Bennison and Coatney (1945) and Corradetti et al. (1966).

Wellde and Sadun (1967) reported a resistance produced in rats and mice by exposure to irradiated P. berghei. Inoculation with parasitized blood exposed to 20 kR stimulated a resistance to a challenge infection with nonirradiated parasites. The peak parasitemias reached in the immunized animals were significantly lower than those of the nonimmunized controls and reduction in parasitemias began much earlier. The acquired resistance induced by irradiated infected erythrocytes could be detected also in a

more susceptible animal such as the mouse. In both rats and mice the degree of acquired immunity was directly proportional to the number of immunizing doses. Fluorescent antibody tests on pooled sera indicated that mice and rats were able to produce a detectable level of antibodies even when the normal development and multiplication of Plasmodia were interrupted by irradiation. Recent studies by Briggs and Wellde (1969) demonstrated a passive protective effect exhibited by serum from mice immunized with irradiated P. berghei blood forms.

Wellde et al. (1969) extended the previous work in which irradiated P. berghei blood forms were used to induce a protective immunity in mice. Even though the ICR strain of mice used was extremely susceptible to the NYU-2 strain of P. berghei (maintained for over 4 years by blood transfers in ICR mice), immunization with irradiated parasitized blood forms induced a resistance which could be detected in some animals for at least 8 months. Mice immunized with irradiated parasitized cells developed a reticulocytosis, a slight decrease in hematocrit values, enlarged spleens and elevated levels of a serum gamma globulin. The increase in spleen weight was probably a direct result of the increased activity involved in the sequestration and destruction of irradiated parasitized cells by this organ. The hypersplenism may have in turn been responsible for both the decrease in hematocrit values and the reticulocytosis since mild hemolytic anemias have been described in rats with hypersplenic conditions (Palmer et al.,

1953). The disparity in blood values between immunized and control mice was apparently not due to subpatent parasitemias which had developed in immunized mice, since repeated subinoculation of blood from these mice produced no infection in recipient animals. Immunized mice which survived the challenging infection apparently underwent a complete cure and remained strongly resistant for a long period of time, since their blood remained free from parasites as determined both microscopically and by subinoculation. This is in agreement with findings reported elsewhere in which true residual immunity without stimulation by surviving parasites was found in both rats (Briggs et al., 1960) and mice (Briggs & Wellde, 1969).

Zuckerman et al. (1967) have reported that antigenic differences between sporozoites and erythrocytic forms may preclude immunization against mosquito induced malaria with blood forms of Plasmodia. The results of Wellde et al. (1969) also suggest that the mechanisms of resistance might be different in animals immunized with sporozoites than in those immunized with blood forms. Furthermore, the work of Richards (1966) indicated that sporozoites inactivated either with ultraviolet light or by drying might be more active agents of immunization than are inactivated erythrocytic parasites. Therefore, several recent studies have been designed to determine whether protective immunity could be produced by the injection of irradiated sporozoites of P. berghei.

Nussenzweig et al. (1967) reported that x-irradiation of sporozoites of P. berghei inactivated these parasites, in certain conditions, so that they no longer produced patent malaria infections detectable by blood smears in mice. Sporozoites were obtained by dissection of the salivary glands of laboratory bred Anopheles stephensi 14 days after feeding on a hamster infected with P. berghei. The amount of irradiation necessary to inactivate sporozoites sufficiently so that patent infection was prevented varied from 6 kR to more than 10 kR. The more viable the batch of sporozoites, the higher the dose of x-irradiation needed to produce this effect. It was further shown that a large percentage of these vaccinated animals were protected against the injection of viable sporozoites. When sufficiently large numbers of irradiated sporozoites were inoculated (7.5×10^4), only 37% of these vaccinated animals became infected on challenge as compared with 90% of their controls. In the rest of the vaccinated animals there was a certain delay of infection, but its invariably lethal outcome was unaltered.

Vanderberg et al. (1968) showed that when 103 mice were each injected with 7.5×10^4 sporozoites of P. berghei irradiated with 8 kR, only 3 developed patent blood infections. The prepatent periods of these animals were longer than those of control mice which had received as few as 1×10^3 nonirradiated sporozoites. After irradiation with 10 to 15 kR, these large dosages of sporozoites (7.5×10^4 per animal) failed to infect any of 29 mice.

In order to detect possible sub-patent infections, blood from mice that did not develop patency after inoculation with irradiated sporozoites was subinoculated into uninfected mice; and in each case, the recipient mice failed to develop parasitemia. Furthermore, mice that did not develop patent infections after injection of irradiated sporozoites failed to develop parasitemia even after they were splenectomized (8 days after sporozoite infection). These conditions are of particular interest in reference to the high degree of immunity developed by many mice which were inoculated with irradiated sporozoites (Nussenzweig et al., 1967). The failure of blood from these animals to produce infections when subinoculated into other animals, and the fact that these immunized animals did not develop patent infections after splenectomy, indicate that immunity could not be considered to have been developed in response to a long standing subpatent blood infection.

Nussenzweig et al. (1969b) reported results obtained from studies designed to determine whether a strict strain and species specificity occur in animals vaccinated with sporozoites, or if a much broader spectrum of protective immunity develops. Mice were immunized by the repeated injection of sporozoites of P. berghei x-irradiated at 8 kR. The initial immunizing dose per mouse was either 5×10^3 or 7.5×10^4 sporozoites of the NK65 strain of P. berghei. Animals immunized in this way were divided into 4 groups and challenged respectively with sporozoites of

P. berghei, blood forms of P. berghei, sporozoites of Plasmodium vinckei and blood forms of P. vinckei. The number of parasites used was large enough to produce close to 100 per cent infection in the control animals, but small enough to provide a sensitive assay of the immunity tested. Animals were challenged 2 to 3 weeks after their last booster injection. The results of these challenges consistently indicated that animals immunized with irradiated sporozoites of P. berghei were effectively protected against viable sporozoites, not only of the homologous species but also of P. vinckei. This cross-protection was substantial because none of the 27 immunized mice developed a patent infection after inoculation with P. vinckei sporozoites which produced 100 per cent patency and 100 per cent mortality in the control animals. The results of the challenge of animals immunized with sporozoites with blood stages of the homologous P. berghei strain did not show a significant degree of protection. The same lack of protection was observed on challenge with blood stages of P. vinckei.

Nussenzweig et al. (1969a) reported that immunization of mice with x-irradiated sporozoites of P. berghei produced, under appropriate circumstances, complete protection against an otherwise 100 per cent lethal sporozoite inoculum. The partial protection obtained with a single immunizing dose was increased with repeated immunizing injections, and by using larger numbers of irradiated sporozoites. A dose of 7.5×10^4 irradiated sporozoites

(parasites obtained from 15-20 infected A. stephensi), was sufficient, when repeated, to completely prevent the development of patent infections upon challenge. The protection was of rather long duration, remaining apparently unchanged for up to 2 months after the last immunizing dose. The degree of protection declined thereafter, but still remained appreciable (± 50% protection) 3 months after the immunization was completed.

Plasmodium falciparum. Sadun et al. (1969) reported a resistance produced in owl monkeys (Aotus trivirgatus) by inoculation with irradiated Plasmodium falciparum. An acquired resistance to infection with P. falciparum developed in owl monkeys after 4 weekly immunizations with parasitized erythrocytes exposed to 25 kR. The resistance developed was sufficient to inhibit the multiplication of the Plasmodia and to permit some animals to survive otherwise lethal challenges with nonirradiated parasites. A less striking degree of acquired immunity was observed following 3 weekly immunizations and no immunity was detected following a single dose. Monkeys were shown to be able to produce a detectable level of antibodies following immunization with irradiated parasites.

Low Temperature Preservation of Babesia

The apparent lability of newly isolated Babesia spp. emphasizes the need for the establishment of stabilates (Irvin &

Brocklesly, 1969). Many factors, such as clonal selection, methods of transmission, type of host animal, and antigenic variation are probably involved in the lability of Babesia spp. (Phillips, 1969).

The establishment of stabilates by freezing is a well recognized procedure in trypanosomiasis research, and has been suggested by Barnett (1964) to workers concerned with Babesia. However, before one can speak of a real "stabilate" (Lumsden & Hardy, 1965) for Babesia, one must be sure that none of the biological properties of the frozen population is altered.

The low temperature preservation of Babesia spp. has recently received attention. Reusse (1956) reported successful preservation of B. canis by slowly cooling infected erythrocytes suspended in an equal volume of normal saline and glycerine so that the final concentration of glycerine in the mixture was 7.5% (v/v). The rate of cooling was 1°C per minute from +5°C down to -20°C and then rapid freezing to storage at -76°C. Blood preserved for 9 weeks was found to be infective following inoculation in a dog. At the Animal Research Institute, Queensland Department of Agriculture and Stock, B. argentina was also found to be infective after deep freezing in bovine blood for 6 weeks (Anon., 1956).

Waddell (1963) reported that B. bigemina survived storage in bovine blood with 11.6% (v/v) glycerol at -79°C for 30 days following uncontrolled freezing, whereas B. bigemina frozen by a controlled method did not survive. For uncontrolled freezing,

the ampoules were immersed in a mixture of solid carbon dioxide and alcohol at -79°C and maintained at -79°C in a vacuum flask by adding crushed solid carbon dioxide 3 times a week. For controlled freezing, the ampoules at 0°C were lowered at a rate of 1°C each 2 minutes to -15°C. The ampoules were then immersed in the deep freezing mixture at -79°C and stored as in the uncontrolled freezing method.

Barnett (1964) reported blood containing B. bigemina with 7%(v/v) glycerol was infective for cattle after storage at -79°C for up to 720 days, the longest period tested. No change in virulence or behavior of the parasite resulted from storage. Three methods of slow reduction of temperature to -79°C were described and all were successful, but in the absence of a method of titrating the number of viable parasites it was not possible to compare the relative efficiency of the 3 methods. In all 3 methods freezing was slow until reaching -18°C. A trial was performed to see whether slow melting would affect the infectivity of the blood and to see how long the organism would remain viable at room temperature once the blood was melted. Two tubes of infected blood were removed from the storage cabinet, and one was melted in warm water, at 37°C and immediately injected into a steer. The other tube was held at room temperature and allowed to melt slowly. After 15 minutes when it was completely melted it was injected s/c into another steer. Both animals became infected. Results of another trial indicated that parasites will survive slow thawing

and will remain viable for at least 1 hour after removal from storage in solid carbon dioxide. Barnett (1964) also reported that porcine blood containing Babesia trautmanni was infective after slow cooling and storage at -79°C for 35 days.

Pipano and Senft (1966) reported that whole blood containing B. bigemina and Babesiella berbera with 15% (v/v) glycerol or glucose in phosphate buffer was infective for calves after storage at -79°C. The incubation periods were nearly identical with those observed after the use of fresh infected blood. No change in virulence of the frozen parasites was noted. The infected blood was dispensed in 10 to 50 ml bottles and incubated for about ½ hour at 4°C. After incubation, the infected blood was stored in a Revco ultra low temperature cabinet at -79°C. Glycerinated blood containing B. bigemina was infective for calves after storage at -70°C for 440 days and B. berbera was infective after 215 days. B. bigemina was preserved in blood containing 15% glucose for 240 days (longest period tested); and B. berbera for 15 days only. Calves inoculated with blood containing B. berbera and 15% glucose after 48 and 174 days of storage failed to react.

Frerichs et al. (1968) reported successful storage of B. caballi and B. equi in liquid nitrogen without loss of virulence. Glycerol was used as a cryoprotective agent, at a final concentration of 11.6% (v/v). The blood-glycerol mixture was stirred magnetically at 5°C for 2 hours, placed in glass vials which were

sealed, cooled to -75°C at a rate of 1 degree C per minute and plunged into liquid nitrogen. One hundred ml aliquots of blood containing B. caballi were removed at 63, 221, 605, and 1,040 days after freezing and injected intravenously into 2-year-old test ponies. The blood containing B. equi was tested 486 and 603 days after freezing. The stored Babesia parasites produced typical signs of equine babesiosis in each of the test ponies. When tested against serums of known titer, antigens prepared from the blood of the last pony in each test series showed the same CF reactions as did antigens prepared at the beginning of the study.

Overdulve and Antonisse (1970) measured the effect of low temperature on B. rodhaini by titration. The titrations were performed with B. rodhaini before and after slow freezing and storage for 42 days at -76°C, either without or with addition of 5 or 10% dimethyl sulfoxide (DMSO). After deep freezing for 42 days at -76°C in 5 or 10% DMSO, only 0.16 to 0.65% of the parasites, counted before deep freezing, remained infective. Addition of 5 or 10% DMSO caused a small loss of infectivity, either because it was toxic or because some parasites lost their ability to multiply in mice. The virulence of the parasites which remained infective, however, was not changed by the concentration of DMSO.

MATERIALS AND METHODS

Experimental Animals

Three-day-old male Holstein calves were obtained from near Facatativa, Colombia and were hand-raised to 3 months of age on the Tibaitata Instituto Colombiano Agropecuario (ICA) experiment station, in an area free of *Boophilus microplus*. The calves were housed for the duration of the experiment in a tick-free environment at the ICA Laboratorio de Investigaciones Medicas Veterinarias (LIMV) in Bogota, Colombia. Insect control was maintained by the periodic application of a residual Dichlorvos* oil base spray in the housing area.

The calves were given a grain ration (see Appendix Table A1) at the rate of 2.7% of their body weight which met the daily nutrient requirements for growth and maintenance of dairy calves suggested by the National Research Council (Anon., 1966). The calves were also given oat silage, salt, and a 1:1 (v/v) mixture of salt and bone meal free choice throughout the experiment.

The calves were screened for the incidence of helminthism and coccidiosis by fecal examination and treated with thiabendazole** and sulfabromomethazine*** before the experiment began.

---

*Vapona, Shell Colombia, S. A., Bogota.

**Thibenzole, Merck Sharp & Dohme Quimica de Colombia, S. A., Bogota and Cali, Colombia.

***Suldur, R. E. Squibb & Sons, Inter-American Corporation, Cali, Colombia.

The calves were vaccinated against foot-and-mouth disease, virus types O and A, Clostridium chauvoei, Clostridium septicum, Pasteurella multocida, and Pasteurella hemolitica before the experiment began.

Splenectomized calves were used for the isolation and separation of B. bigemina, preparation of irradiated and challenge inocula, subinoculation for the detection of subpatent parasitemia and production of antigens for the CF test. Splenectomy was carried out under general anesthesia using 1 ml per kg of body weight of an aqueous solution of chloral hydrate (42.50 g/l), pentobarbital (9.72 g/l), and magnesium sulfate (11.26 g/l).* The spleen was exposed through a left lateral incision in the paralumbar fossa and after ligating the splenic artery and vein, the spleen was removed. Blood smears and CF reactions for Babesia and Anaplasma were determined weekly for at least 5 weeks after splenectomy and prior to inoculation, to insure that they did not carry a latent infection with Babesia or Anaplasma.

Experimental Organisms

The B. bigemina used in the following studies was obtained from a splenectomized calf which had an acute tick-transmitted infection of B. bigemina. A 4-month-old splenectomized calf was

---

*Equi-Thesin, Jensen-Salsbery Laboratories, Kansas City, Mo. 64142.

transported by airplane from the ICA-LIMV laboratories in Bogota to the northern coastal area of Colombia and placed on a tick-infested pasture at the Turipana ICA experiment station near Monteria.

Daily thin films were made with blood obtained from the jugular vein using 1.3 g/l of disodium ethylenediamine tetraacetate (EDTA) as an anticoagulant. The films were fixed in absolute methanol and stained with a 1 to 20 solution of Giemsa* for 20 minutes, using phosphate buffered water (pH 7.0-7.1) containing 0.01% alkyl phenoxy polyethoxy ethanol** (APPE) to prepare the stain solution. Stock solutions of M/15 Na_2HPO_4, M/15 NaH_2PO_4, and 10% APPE were prepared and stored in separate glass stoppered bottles. Fresh buffered water containing 0.01% APPE was prepared weekly by using 39 ml of M/15 NaH_2PO_4, 61 ml of M/15 Na_2HPO_4, 1 ml of 10% APPE, and 899 ml of distilled water. An electric pH meter*** was used to determine the pH of the freshly prepared buffered water containing APPE. The films were examined microscopically, using a microscope with an oil immersion objective, for 30 minutes in

---

*Giemsa Stain. Gradwohl Laboratories, 3514 Lucas Avenue, St. Louis, Mo. 63155.

**Triton X-100. Rohm and Hass, Independence Mall West, Philadelphia, Pa. 19105.

***Beckman Expandometic Model 76. Beckman Instruments, Inc., Scientific Instruments Division, Fullerton, California 92634.

cases when the first appearance of Babesia was thought to be imminent. The films were examined about 3 to 6 mm from the end of the film and transversed from one side of the film to the other to give a constant and representative sample. After B. bigemina was found in blood smears, 180 ml of blood were withdrawn from the jugular vein of the splenectomized calf, using 1.3 g/l of dipotassium EDTA as an anticoagulant. The blood was transported by airplane to the ICA-LIMV laboratories in Bogota for the separation of B. bigemina from B. argentina, B. major, and Anaplasma marginale.

The method of separation of B. bigemina from B. argentina, B. major, and A. marginale involved rapid passage through 5 splenectomized calves and was based on that used by Sergent et al. (1927) and Callow and Hoyte (1961a). The first calf was inoculated with blood carrying several different organisms, and subsequent subinoculations were done soon after blood smears from each calf were found to be positive for B. bigemina. Blood for subinoculation was collected from the jugular vein without using an anticoagulant and each passage was carried out immediately by injecting the blood into the jugular vein of the next splenectomized calf.

The drugs used to treat the Babesia infections were trypan blue and 4,4' -diamidino-diazoaminobenzene diaceturate.* Trypan

---

*Ganaseg. Squibb, Cali, Colombia.

blue was used for suppressing the B. bigemina infection without interfering with the potential B. argentina infection.

A frozen stabilate of B. bigemina was made using a modification of the method reported by Pipano and Senft (1966). Blood containing 2.5% B. bigemina parasitized erythrocytes was collected from the fifth splenectomized calf at the acute stage of the disease by venipuncture using 1.3 g/l disodium EDTA as an anticoagulant. Glycerol was used as a cryoprotective agent, at a final concentration of 11.6%. Fifty ml of the blood-glycerol mixture were dispensed in 60 ml plastic narrow mouth bottles with screw caps*, and incubated for 30 minutes at 4°C. After incubation, the blood was stored in a dry ice cabinet at -79°C.

Preparation of Irradiated and Challenge Inocula

Blood containing B. bigemina was collected from splenectomized calves during the acute stage of babesiosis after inoculation with 50 ml of the frozen stabilate of B. bigemina. Although calves were inoculated or challenged at different times, each inoculum was prepared from splenectomized calves previously inoculated with a standard dose of the same B. bigemina infected blood. Blood containing B. bigemina was collected from the donor splenectomized calves during the acute stage of the disease by venipuncture using 1.3 g/l of dipotassium EDTA as an anticoagulant.

---

*Kimble, Owens-Illinois, Toledo, Ohio 43601.

The number of B. bigemina parasitized erythrocytes per ml of blood was determined from an estimate of the percentage of erythrocytes parasitized and the erythrocyte count.

For irradiation treatment, 120 ml of inoculum were divided equally between 2 plastic Petri dishes, 1.3 cm deep and 9.1 cm in diameter. The depth of inoculum in each Petri dish was approximately 1.0 cm. The inoculum in one Petri dish was exposed to the desired amount of radiation using a cobalt-60 teletherapy unit*. This 3129-c source delivered a dose rate of approximately 200 Rads/min at an exposure distance of 50 cm. The dose rate was determined by ferrous chemical dosimetry and periodic verification of the dose rate was carried out by means of a dosimeter**. The inoculum in the other Petri dish was placed in an adjacent room for the duration of the irradiation and used as a nonirradiated control.

Experimental Procedures

Five experiments were performed to study the effects of various gamma radiation dosages on the infectivity and immunogenicity of erythrocytic stages of B. bigemina in cattle. In experiment

---

*Eldorado 8. Atomic Energy of Canada Limited: Commercial Products, P. O. Box 93, Ottawa, Canada.

**Victoreen r-Meter Mod. No. 570. Victoreen Instrument Division, 10101 Woodland Ave., Cleveland, Ohio 44104.

I, 8 three-month-old calves were divided into 4 groups of 2 calves each. Three groups of calves were given one intravenous inoculation of blood containing 1 x 10^9 B. bigemina parasitized erythrocytes previously exposed to radiation doses of 22, 26, and 30 kRad. One calf in the control group was given one intravenous inoculation of nonirradiated blood containing 1 x 10^9 B. bigemina parasitized erythrocytes and the other control calf received one intravenous inoculation of a similar volume of nonparasitized blood previously exposed to a radiation dose of 30 kRad. All calves in experiment I were challenged intravenously with blood containing 1 x 10^{10} B. bigemina parasitized erythrocytes 7 weeks after the last inoculation.

In experiment II, 6 three-month-old calves were divided into 3 groups of 2 calves each. Two groups of calves were given one intravenous inoculation of blood containing 1 x 10^9 B. bigemina parasitized erythrocytes previously exposed to radiation doses of 36 and 42 kRad. The groups of calves that failed to show parasitic multiplication and reinvasion of erythrocytes were given a second inoculation in a similar manner 14 days after the first inoculation to enhance any immunizing effect that the irradiated parasites might have had. One calf in the control group was given one intravenous inoculation of nonirradiated blood containing 1 x 10^9 B. bigemina parasitized erythrocytes and the other control calf received 2 intravenous inoculations at a 14 day interval of a similar volume of nonparasitized blood

previously exposed to a radiation dose of 42 kRad.

In experiment III, 4 three-month-old calves were divided into 2 groups of 2 calves each. The 2 groups of calves were given one intravenous inoculation of blood containing 1 x 10^9 B. bigemina parasitized erythrocytes previously exposed to radiation doses of 36 and 42 kRad. The inoculation schedules in experiments II and III were arranged so that all calves received their final inoculation on the same day. All calves in experiments II and III were challenged intravenously with blood containing 1 x 10^{10} B. bigemina parasitized erythrocytes 3 weeks after the last inoculation.

In experiment IV, 31 four-month-old calves were divided into 4 experimental groups of 4 calves each and 5 control groups of 3 calves each. The 4 experimental groups of calves were given one intravenous inoculation of blood containing 1 x 10^{10} B. bigemina parasitized erythrocytes previously exposed to radiation doses of 24, 36, 48, and 60 kRad.

The first control group of calves were given one intravenous inoculation of nonirradiated blood containing 1 x 10^{10} B. bigemina parasitized erythrocytes. To evaluate the possibility that some reduction in parasitemia might be the result of a reduction in the number of viable parasites injected rather than an attenuation of the parasites injected, the second and third control groups of calves received on intravenous inoculation of nonirradiated blood containing 1 x 10^7 and 1 x 10^4 B. bigemina parasitized

erythrocytes, respectively. To test the hypothesis that living erythrocytic stages of B. bigemina exposed to a radiation dose sufficient enough to prevent progressive parasitemias are more immunogenic than non-living parasites, the fourth control group of calves were given one intravenous inoculation of blood containing 1×10^{10} B. bigemina parasitized erythrocytes previously heat inactivated in a water bath at 56°C for 30 minutes. The fifth control group of calves received one intravenous inoculation of a similar volume of nonparasitized blood previously exposed to a radiation dose of 36 kRad. All calves in experiment IV were challenged intravenously with blood containing 1×10^{10} B. bigemina parasitized erythrocytes 4 weeks after their last inoculation.

Experiment V was undertaken to study the effect of freezing on the immunogenicity of irradiated erythrocytic stages of B. bigemina. An aliquot of blood containing B. bigemina parasitized erythrocytes previously exposed to a radiation dose of 60 kRad for the fourth experimental group in experiment IV was frozen using the method previously described for the preservation of B. bigemina. Four 4-month-old calves were given one intravenous inoculation of irradiated blood containing 1×10^{10} B. bigemina parasitized erythrocytes previously frozen for 48 hours. The inoculated calves in experiment V were challenged intravenously with blood containing 1×10^{10} B. bigemina parasitized erythrocytes 26 days after the last inoculation with an aliquot of the same challenge inoculum used in experiment IV and on the same day as in experiment IV.

Assessment of Reactions

Parasitemia

Combination thin and thick blood films. The calves were examined daily for the presence of parasitized erythrocytes by the use of combination thin and thick blood films. The blood films were made with blood obtained from the jugular vein using 1.3 g/l of disodium EDTA as an anticoagulant. The thin blood film was extended over ½ the length of the microscope slide and rapidly dried. The method used for the preparation of the thick film on the opposite end of the microscope slide was based on a technique described by Mahoney and Saal (1961).

The thin blood film was fixed with absolute methyl alcohol. The slide was held in a slanted position with the thin film down and a few drops of absolute methyl alcohol placed on the thin film. To prevent alcohol or alcohol fumes from contacting the thick film, the slide was placed in a vertical position with the thick film up until the methyl alcohol had evaporated. The slides were then placed in a staining rack* and kept at this stage for further processing.

Just before staining, the slides were placed in an incubator at 65°C for 45 minutes for final drying of the thick films. Final

---

*Staining Rack Model S7680. Scientific Products, Division of American Hospital Supply Corporation, 1210 Leon Place, Evanston, Illinois 66201.

drying was required to ensure that the thick films did not wash off during the staining process. To prevent deterioration of the thick films, the combination thin and thick films were stained soon after final drying.

The method used for the staining of the combination thin and thick films was based on a staining technique described for Plasmodium spp. in man (Anon., 1970). The thin and thick films were placed in a 1 to 50 solution of Giemsa stain for 45 minutes to lyse the unfixed erythrocytes in the thick film and to stain the blood films. Phosphate buffered water (pH 7.0-7.1) containing 0.01% APPE was used to prepare the stain solution.

Immediately following staining, the thin and thick films were rinsed briefly by dipping the slides in a staining dish filled with buffered water (pH 7.0-7.1) containing 0.01% APPE. A rubber band was placed around the staining rack to hold the slides in place. The staining rack was placed in a vertical position with only the thick film immersed in the same buffered water containing APPE for an additional 3 to 5 minutes. The slides were dried in the vertical position with the thick film down.

For confirmation of Babesia parasitemia on thick films, it was necessary to resolve the objects as having a red nucleus and blue cytoplasm and to find some parasites that were typically pyriform and arranged in pairs. An estimate of the number of parasites per mm^3 of blood on thick films was made from the number

of parasites seen on 20 fields, and from an estimate of the number of leukocytes present on 20 fields, and from an estimate of the number of leukocytes per mm^3 of blood. The number of parasites per mm^3 of blood was then converted to percent parasitized erythrocytes from an estimate of the number of erythrocytes per mm^3 of blood.

Subinoculations. Subinoculations were performed the day of challenge from 4 groups in experiment IV which were inoculated with parasitized erythrocytes, but failed to demonstrate detectable parasitemias to determine whether subpatent parasitemia had resulted. Subinoculations from the 4 groups were accomplished by collecting 100 ml of blood from each calf in each group by venipuncture using 1.3 g/l disodium EDTA as an anticoagulant and injecting the blood intravenously by group into 4 splenectomized calves.

Packed Cell Volume

Daily packed cell volumes were determined by the microhematocrit method with blood obtained from the jugular vein using 1.3 g/l of disodium EDTA as an anticoagulant. Plain capillary tubes 75 mm x 1.2 mm were filled to approximately 1 cm from the end and the vacant end of the tube was sealed by holding it in a flame of a high temperature gas burner. The filled capillary tubes were

then centrifuged* for 5 minutes at 11,500 rpm and the packed cell volumes read directly from a graphic reader**.

Rectal Temperatures and Body Weights

Daily rectal temperatures were measured each morning throughout the experiment. All calves were weighed*** weekly following a 12 hour withdrawal from feed.

Babesial Complement Fixing Antibody Levels

General. Determination of babesial CF serum antibody levels was performed by a microtiter procedure similar to that described by Hidalgo and Dimopoullus (1967). Serum samples were collected from all calves twice a week for the CF microtiter procedure. Whole blood was collected by jugular puncture, allowed to clot, and serum recovered after centrifugation**** at 2,500 rpm for 20 minutes.

The CF microtiter procedure was conducted in polished transparent plastic trays containing 12 rows of 8 wells each. Reagents

---

*International Micro-Capillary Centrifuge, Model MB. International Equipment Company, Needham Hts., Mass. 02194.

**International Micro-Capillary Reader. International Equipment Company, Needham Hts., Mass. 02194.

***Ranger Balance, Model SF1. Ranger Marting Mfg., Washington C.H., Ohio 43160

****International Centrifuge, Size 2, Model K. International Equipment Co., Needham Hts., Mass. 02194.

except serums were added to wells with dropping pipettes* which delivered 0.025 ml per drop. Serums were transferred to the wells in the test tray and serially diluted with the microloops** which delivered 0.025 ml. Incubation of trays was conducted at 37°C after being covered with an airtight plate sealer to prevent excessive evaporation of reagents.

Veronal buffer was used to dilute all reagents in the CF microtiter procedure. It was prepared and stored as described in the standard anaplasmosis CF procedure (Anon., 1958). Ovine erythrocytes for the hemolytic system were collected, stored, and washed as previously described (Anon., 1958). For conducting the CF microtiter procedure, 2.0% suspensions of ovine erythrocytes were prepared as described in the standard anaplasmosis CF procedure (Anon., 1958).

Hemolysin titration. The following reagents were prepared to titrate hemolysin: (1) 2% ovine erythrocytic suspension, (2) 1:25 dilution of a commercially produced complement***, and (3) 1:1,000 dilution of a commercially produced hemolysin****. The

---

*Microtiter Pipette Dropper. Cooke Engineering Company, Medical Research Division, 900 Slaters Lane, Alexandria, Va. 22314.

**Microtiter Loops. Cooke Engineering Company, Medical Research Division, 900 Slaters Lane, Alexandria, Va. 22314.

***TBL Lyophilized Guinea Pig Complement. Texas Biological Lab. Inc., P.O. Box 722, Fort Worth, Texas 76101.

****Sycco Sheep Cell Hemolysin. The Sylvana Co., Millburn, New Jersey 07041.

1:1,000 dilution of hemolysin was further diluted and titrated according to the scheme previously described (Anon., 1958). For the preparation of the hemolytic system, equal amounts of the 2% suspension of ovine erythrocytes and diluted titrated hemolysin were mixed and stored at 4-6°C for 1 day before using. A unit of hemolysin was defined as the highest dilution producing complete hemolysis of an equal volume of a 2% ovine erythrocyte suspension within 45 minutes at 37°C in the presence of an excess of complement.

Complement titration. The following reagents were prepared to titrate the complement: (1) 2% ovine erythrocytic suspension, (2) hemolysin dilution (2 units/ml), and (3) 1:25 dilution of commercially produced complement. The complement was further diluted and titrated according to the scheme previously described (Anon., 1958). A unit of complement was defined as the highest dilution producing complete hemolysis of a equal volume of 2% ovine erythrocytic suspension in the presence of an equal volume of hemolysin (2 units/ml) within 45 minutes at 37°C.

Preparation of antigen. Blood containing 23% *B. bigemina* parasitized erythrocytes was collected by carotid artery canulation from a splenectomized calf during the acute stage of babesiosis after inoculation with 50 ml of the frozen stabilate of *B. bigemina*. Blood was collected in sterile 2 l Erlenmeyer flasks using 1.3 g/l disodium EDTA as an anticoagulant. After collection, the blood was placed in a refrigerator for 3 hours at 4-6°C. The plasma was

removed after centrifugation* at 2,500 rpm (1020 xg) for 20 minutes at 2-4°C, and the erythrocytes were washed twice with sterile physiological saline at 4-6°C.

Babesia bigemina antigens for the CF microtiter procedure were prepared from the washed erythrocytes by a method described by Mahoney (1967c). The washed erythrocytes were lysed with 10 volumes of 0.35% sodium chloride and the mixture was centrifuged at 5,600 rpm (5,000 xg) for 30 minutes at 2-4°C. The sediment was washed and resuspended in an equal volume of the same diluent and examined microscopically in Giemsa stained smears. Distilled water extracts were prepared by mixing one volume of parasite suspension with 2 volumes of distilled water, shaking vigorously for 2 minutes, and then centrifuging at 5,600 rpm (5,000 xg) for 30 minutes at 4°C. The supernatant fluids were numbered serially in order of collection, placed in ampoules in 3 ml portions and stored in a dry ice cabinet at -79°C. The third extract was strongly antigenic and was the B. bigemina antigen used in the CF microtiter procedure.

Antigen titration. The following reagents were prepared to titrate the antigen: (1) 2% ovine erythrocytic suspension, (2) inactivated positive serums (3) inactivated negative serums, (4)

---

*Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge. Ivan Sorvall Inc., Newtown, Conn. 06470.

hemolysin (2 units/ml), (5) complement (2 units/ml), and (6) antigen dilution of an approximate strength that would cause complete CF within the range of the titration procedure. Antigens were titrated against a group of 8 positive and 2 negative reference serums according to the scheme previously described (Anon., 1958). The positive serums were from animals that had recovered from blood-induced infections. The serums were taken from 1 to 4 months after infection and were inactivated in a water bath at 56°C for 30 minutes. A unit of antigen was defined as the highest dilution causing complete fixation of an equal volume of complement (2 units/ml) in the presence of the weakest positive reference serum diluted 1:5.

Complement fixation microtiter procedure. A 2% ovine erythrocytic suspension and dilutions of antigen, complement, and hemolysin each containing 2 units/ml were prepared to conduct the microtiter procedure. In addition, a 1:2.5 dilution of each serum sample was prepared and inactivated in a water bath at 56°C for 30 minutes. Titrations in the trays were prepared by first adding 0.025 ml of buffer to each of 8 wells of 1 row for each sample. A 0.025 ml loopful of the 1:2.5 dilution of serum was added to the first well and serially diluted through the eighth well. A 0.025 ml drop of antigen and 0.025 ml drop of complement were then added to wells 1 through 8. The serums being tested were then allowed to react with the antigen and complement for 1 hour in a

water bath at 37°C. Subsequently, 0.05 ml of the hemolytic system consisting of an equal volume of diluted hemolysin and 2% ovine erythrocytic suspension was added and the trays were incubated for 1 more hour. Serum controls on all serums tested were prepared and read in tubes according to the scheme previously described (Anon., 1958).

Positive and negative serum standards and antigen, complement, and hemolytic system controls also were prepared. The antigen control consisted of 0.025 ml of complement, 0.025 ml of antigen, 0.025 ml of buffer, and 0.05 ml of hemolytic system. The complement control consisted of 0.025 ml of complement, 0.05 ml of buffer, and 0.05 ml of hemolytic system. The hemolytic system control consisted of 0.075 ml of buffer and 0.05 ml of hemolytic system. After final incubation, trays were stored 2 hours at 4°C to allow unhemolyzed cells to form sediments in the bottom of the wells. Reactions were read by placing the transparent trays on a concave mirror stand. The titer of the serum was considered to be the highest dilution having complete fixation.

RESULTS

Separation of B. bigemina from B. argentina, B. major, and A. marginale

The splenectomized calf placed on a tick-infested pasture at the Turipana ICA station was found to be heavily infested with B. microplus ticks in the larval stage within 3 days, ticks in the nymphal stage within 8 days, and ticks in the adult stage within 14 days. After 16 days, the body temperature of the calf rose to 40.4°C and B. argentina was found in thin blood smears stained with Giemsa. Two days later, B. bigemina was found in blood smears. The results of the rapid passages of B. bigemina through 5 splenectomized calves to separate B. bigemina from contaminating organisms are summarized in Table I.

Babesia bigemina was found in blood smears from the first calf after 36 hours. A B. argentina infection, which developed in the first calf 4 days after the inoculation with blood, was treated with 4,4' -diamidino-diazoaminobenzene diaceturate, 3 mg/kg for 5 days. Anaplasma marginale was present in smears from the first calf 44 days after inoculation.

Babesia bigemina was found in blood smears from the second calf after 30 hours. A B. argentina infection, which developed in the second calf 4 days after the inoculation with blood, was treated with 4,4' -diamidino-diazoaminobenzene diaceturate, 3 mg/kg for the following 2 days. The second calf died from babesiosis 1

Table 1. The Separation of B. bigemina from B. argentina, B. major, and A. marginale by Rapid Passage through 5 Splenectomized Calves.

Passage Level	Inoculum	Incubation	Parasite	Time of Subinoculation
1	180 ml I. V. from naturally infected calf	36 hours	B. bigemina	37½ hours
2	12 ml I.V. from No. 1	30 hours	B. bigemina	31 hours
3	12 ml I.V. from No. 2	24 hours	B. bigemina	24 hours
4	12 ml I.V. from No. 3	37½ hours	B. bigemina	37½ hours
5	12 ml I.V. from No. 4	57½ hours	B. bigemina	----------

day after the last treatment.

The third calf was found to be positive for B. bigemina 24 hours after inoculation with 12 ml of blood from the second calf. The third calf was subsequently treated with an intravenous injection of 20 ml of 1% trypan blue, which suppressed the B. bigemina infection. Babesia argentina and B. major infections, which developed in the third calf 15 days after inoculation with blood, were treated the following day with 4,4' -diamidino-diazoaminobenzene diaceturate, 3 mg/kg. The third calf died from babesiosis 1 day after treatment.

The fourth calf was found to be positive for B. bigemina 37½ hours after inoculation with 12 ml of blood from the third calf. The procedure of treating the fourth calf with trypan blue was repeated.

Babesia bigemina was found in blood smears from the fifth calf after 57½ hours and within an hour, 12 ml of blood were taken for intravenous passage into an intact calf. A frozen stabilate of B. bigemina was made from the fifth calf 4 days after inoculation when the parasitemia had reached 2.5%. The day following freezing, 50 ml of the frozen stabilate was inoculated intravenously into a second intact calf.

The first intact calf was found to be positive for B. bigemina 2 days after inoculation. The second intact calf was found to be positive for B. bigemina 5 days after inoculation. The intact calves did not require treatment with trypan blue. Babesia

argentina, B. major, and A. marginale were not found in any smear from either of the 2 intact calves nor in any smear from the fourth and fifth splenectomized calves for 2 months after inoculation. Babesia argentina and B. major were therefore eliminated as contaminating organisms after 4 passages. The second and third splenectomized calves died of babesiosis which made it impossible to estimate at which point A. marginale failed to be passaged. Photographs of B. bigemina on thin and thick blood films are shown in Figures 1 and 2, respectively. Babesia bigemina used for the preparation of antigens for the CF microtiter procedure is shown in Figure 3.

Experiment I

The first experiment was undertaken to determine the effect of gamma radiation dosages of 22, 26, and 30 kRad on the infectivity and immunogenicity of B. bigemina in calves.

The results summarized in Figures 4 to 8 and Table 2 show that calves infected with 1×10^9 B. bigemina parasitized erythrocytes exposed to dosages up to and including 30 kRad developed progressive parasitemias which were delayed in comparison to the calves inoculated with nonirradiated B. bigemina parasitized erythrocytes. The average prepatent periods were 7½, 9, and 10 days for calves infected with 1×10^9 B. bigemina parasitized erythrocytes exposed to 22, 26, and 30 kRad, respectively. The average prepatent periods for calves infected with nonirradiated

Figure 1. Babesia bigemina parasitized erythrocytes on thin blood film. Giemsa. x1330.

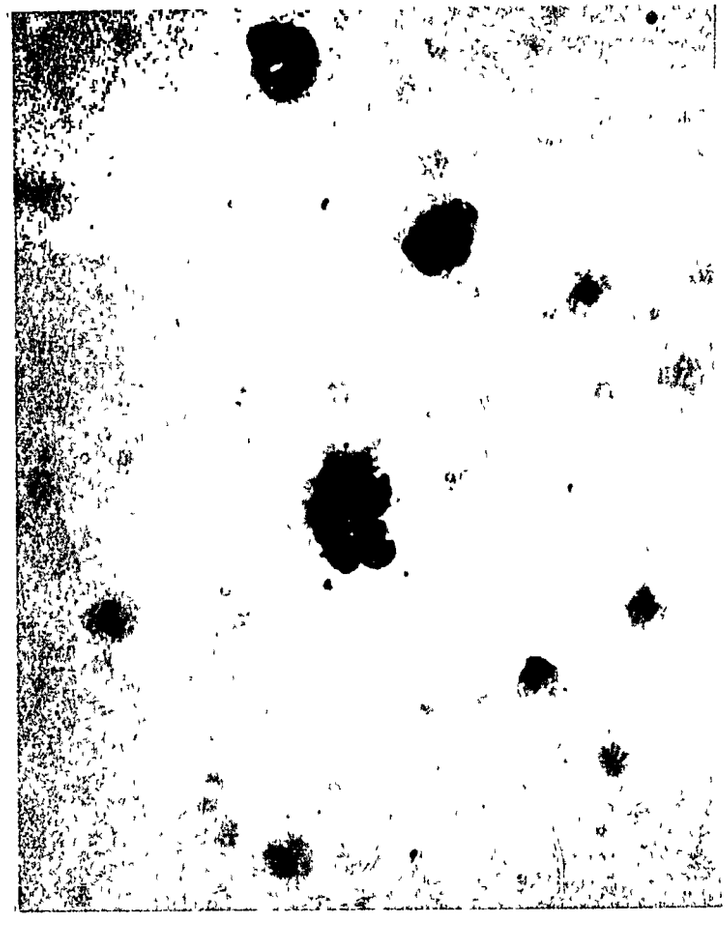


Figure 2. Babesia bigemina parasites on thick blood film. Giemsa. x1330.

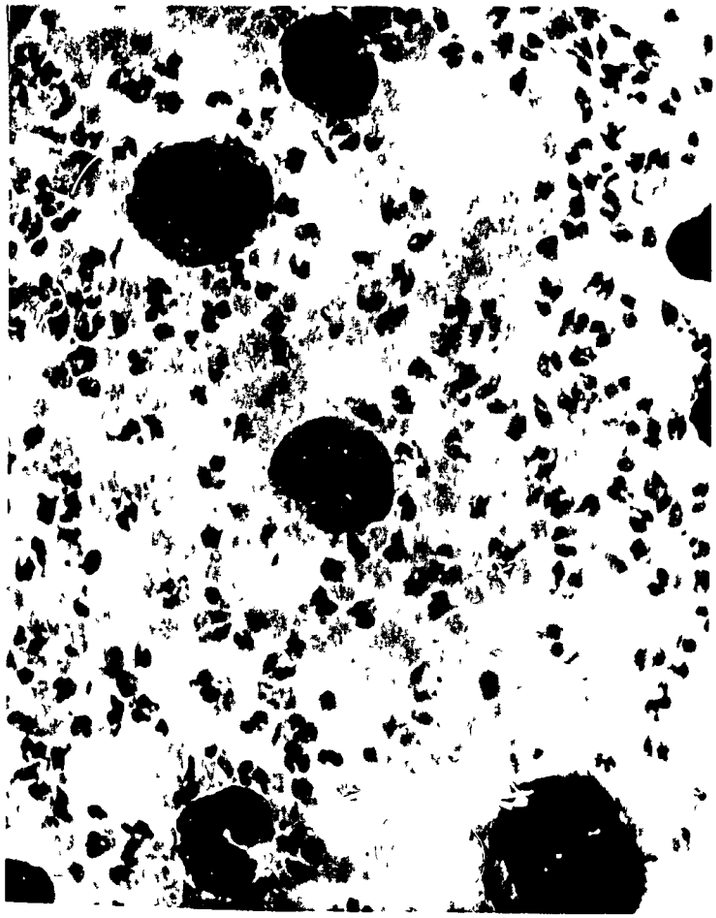


Figure 3. Babesia bigemina used for the preparation of antigens for the CF microtiter procedure on thick film. Giemsa. x1330.

Figure 4. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 2 calves inoculated (I) with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 22 kRad, and challenged (C) 7 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes. Group means are represented by horizontal lines and ranges of the means are represented by vertical lines in this figure and in figures which follow.

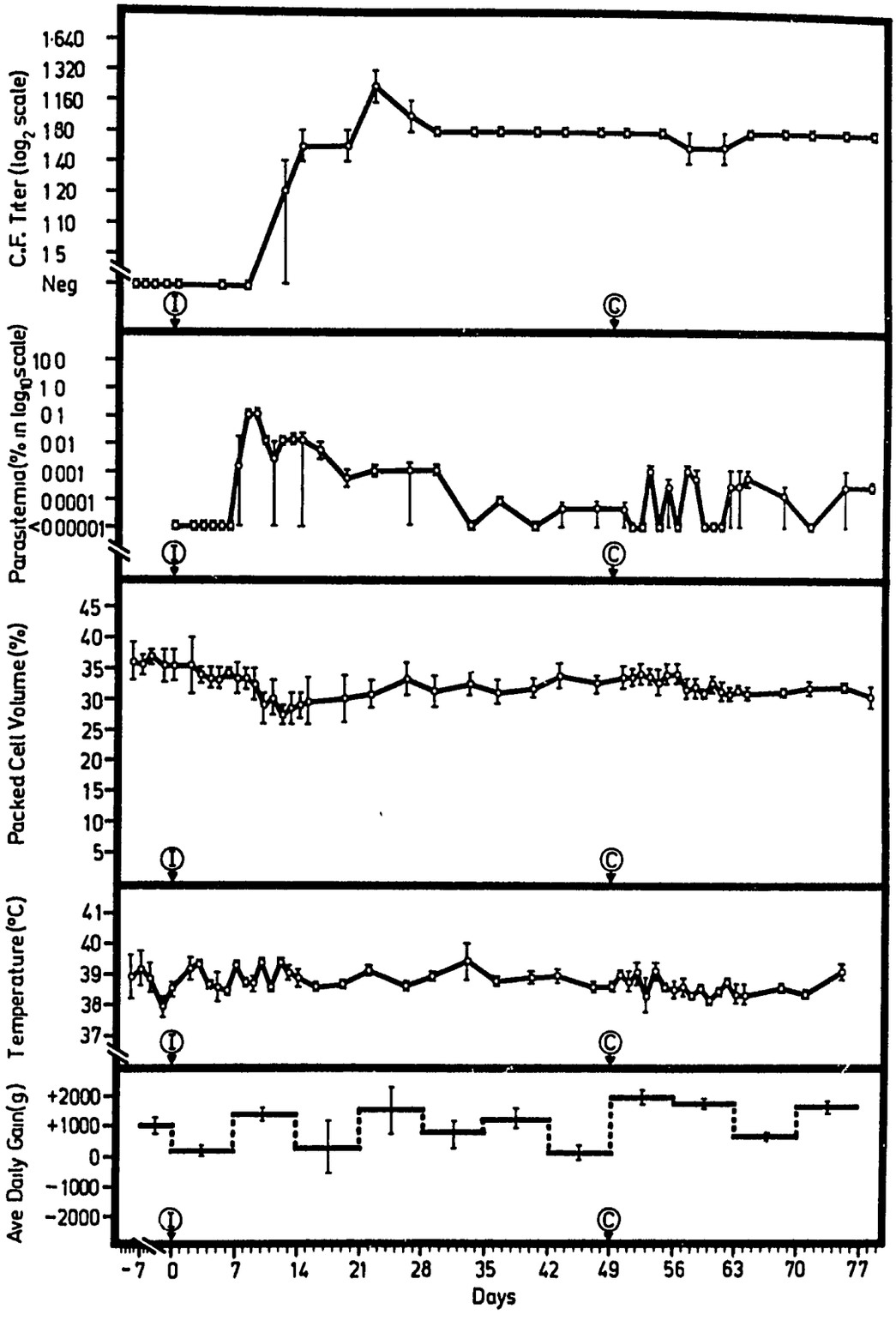

C.F. Titer (log2 scale)
1·640
1 320
1 160
180
140
120
110
15
Neg
Parasitemia(% in log10 scale)
100
10
01
001
0001
00001
<000001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave Daily Gain(g)
+2000
+1000
0
−1000
−2000
I
C
-7
0
7
14
21
28
35
42
49
56
63
70
77
Days

Figure 5. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 2 calves inoculated (I) with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 26 kRad, and challenged (C) 7 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes.

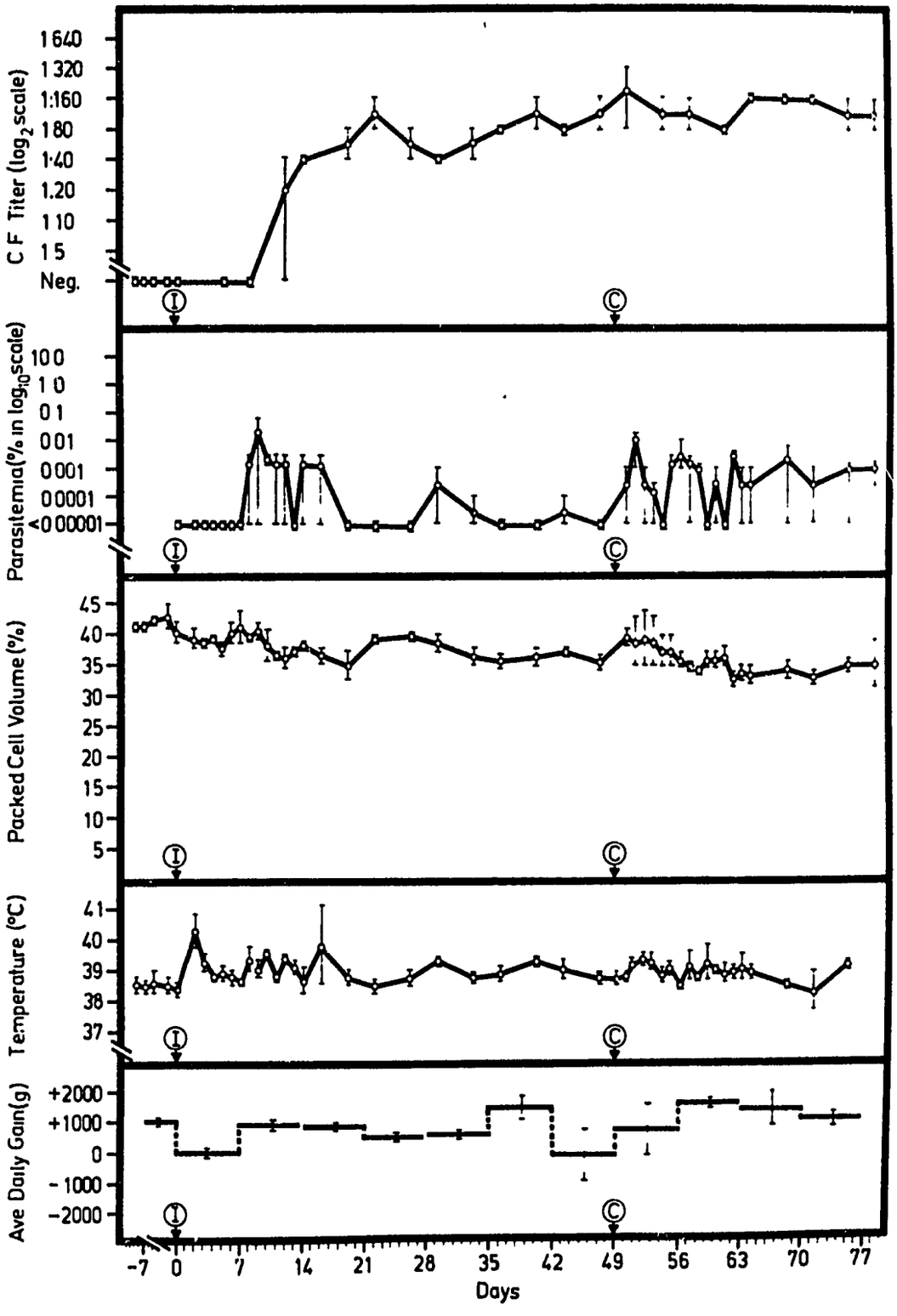

C F Titer (log2 scale)
1640
1320
1:160
180
1·40
1.20
110
15
Neg.
Parasitemia(% in log10 scale)
100
10
01
001
0001
00001
<000001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave Daily Gain(g)
+2000
+1000
0
-1000
-2000
I
C
-7
0
7
14
21
28
35
42
49
56
63
70
77
Days

Figure 6. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 2 calves inoculated (I) with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 30 kRad, and challenged (C) 7 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes. One calf (*) died 34 days after inoculation with chronic bacterial pneumonia.

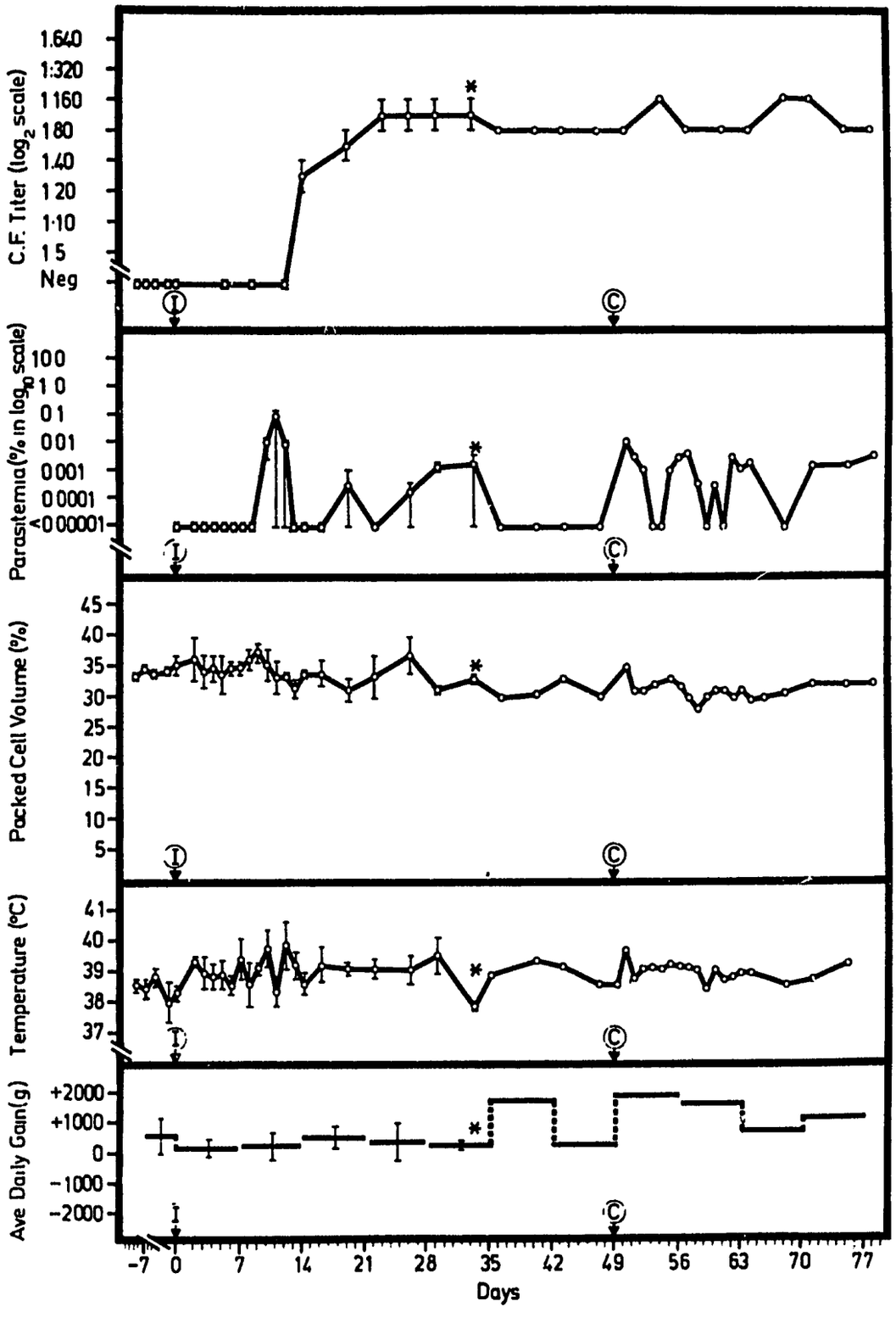

C.F. Titer (log2 scale)
1.640
1:320
1 160
1 80
1.40
1 20
1·10
1 5
Neg
Parasitemia (% in log10 scale)
100
1 0
0 1
0 01
0 001
0 0001
0 00001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave Daily Gain (g)
+2000
+1000
0
−1000
−2000
I
C
*
−7
0
7
14
21
28
35
42
49
56
63
70
77
Days

Figure 7. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 1 calf inoculated (I) with 1 x 10^9 nonirradiated B. bigemina parasitized erythrocytes, and challenged (C) 7 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes.

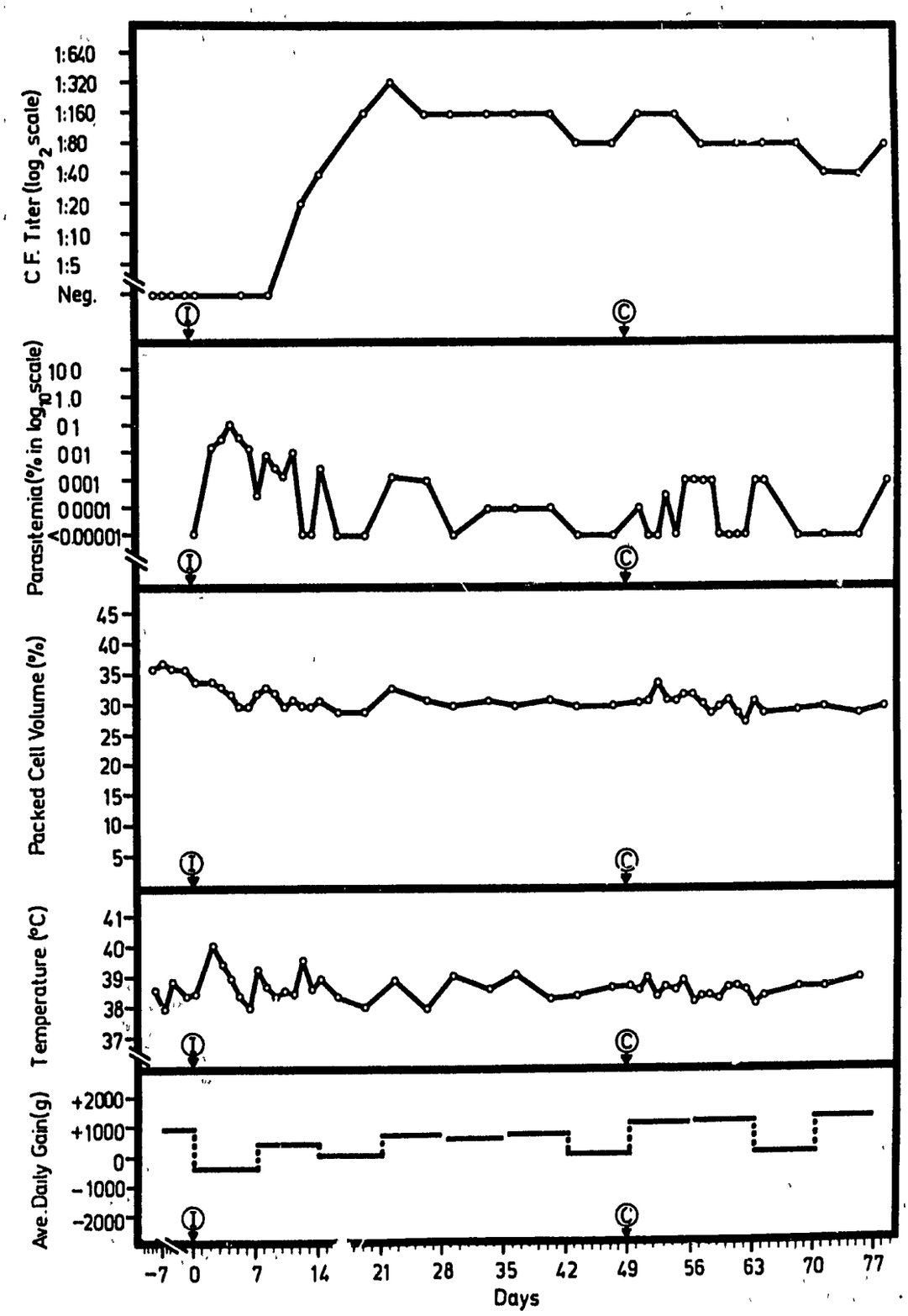

C.F. Titer (log2 scale)
1:640
1:320
1:160
1:80
1:40
1:20
1:10
1:5
Neg.
Parasitemia (% in log10 scale)
10.0
1.0
0.1
0.01
0.001
0.0001
0.00001
<0.00001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave. Daily Gain (g)
+2000
+1000
0
-1000
-2000
T
C
-7
0
7
14
21
28
35
42
49
56
63
70
77
Days

Figure 8. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 1 calf inoculated (I) with 12 ml of normal blood exposed to 30 kRad, and challenged (C) 7 weeks later with 1×10^{10} B. bigemina parasitized erythrocytes.

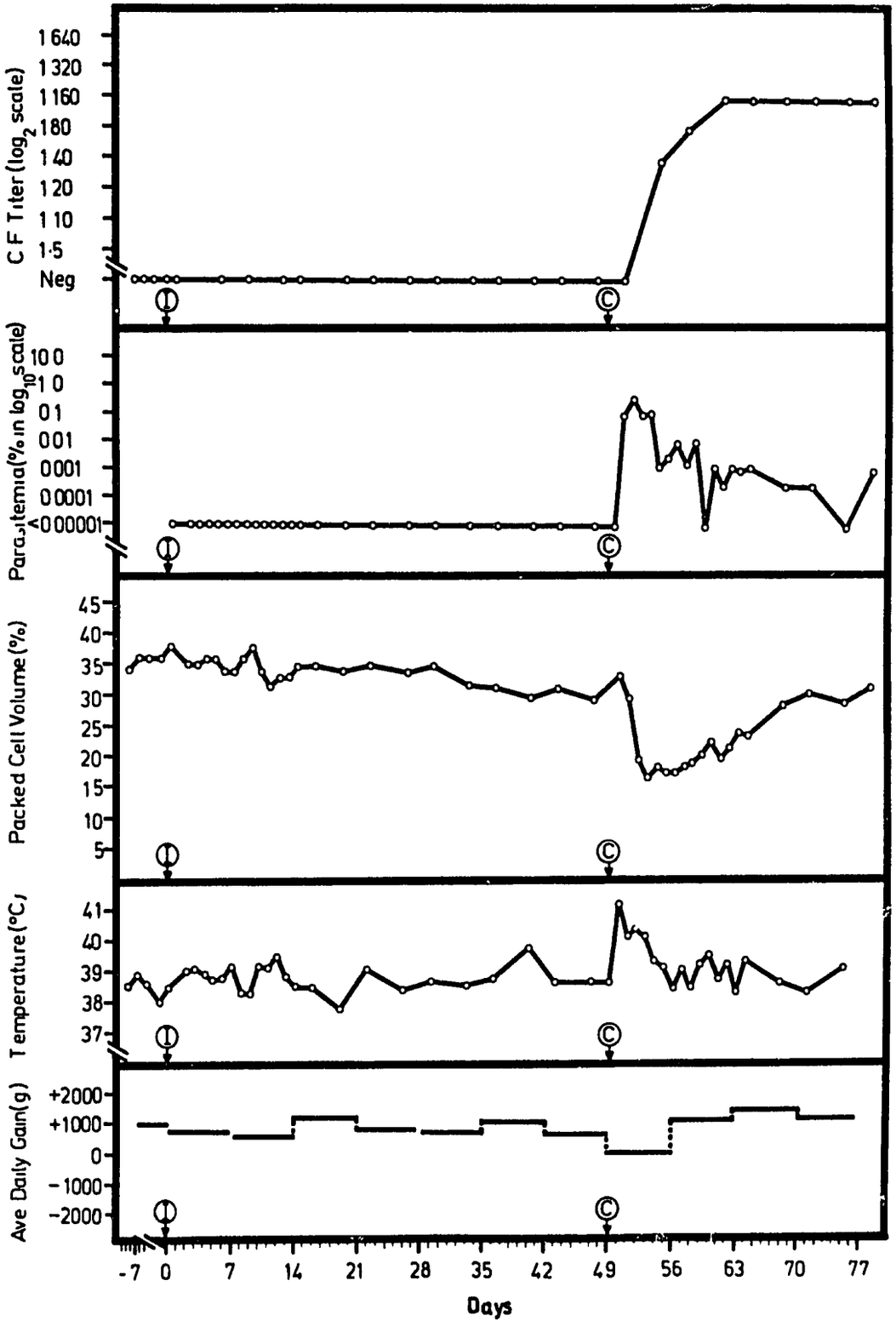

C F Titer (log2 scale)
1 640
1 320
1 160
180
140
120
110
1·5
Neg
Parasitemia (% in log10 scale)
100
10
01
001
0001
00001
<00001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave Daily Gain (g)
+2000
+1000
0
-1000
-2000
I
C
-7
0
7
14
21
28
35
42
49
56
63
70
77
Days

Table 2. Characteristics of *Babesia bigemina* Infections Produced with Infected Blood Subjected to Different Radiation Doses. Summary of Experiments I, II, III and IV.

Exp. No.	Radiation Dose of Inoculum In Kilorads	No. of Parasitized Erythrocytes Inoculated	Reaction After Inoculation of Irradiated B. bigemina Parasitized Erythrocytes[a]: No. Calves with Parasitemia / No. Calves Inoculated[b]	Prepatent Periods in Days	Maximum % Parasitized Erythrocytes	Maximum Temperature in °C	Minimum Packed Cell Volume in %	Maximum Complement Fixation Titer	Average Daily Gain in g/day	Deaths
IV	0 (controls)	1×10^{10}	3/3	2.0 (2-2)[c]	3.2 (0.4-8.0)	41.5 (41.3-41.6)	18 (13-28)	1:320 (1:160-1:640)	423 (71-821)	0
I + II	0 (controls)	1×10^{9}	2/2	2.0 (2-2)	0.29 (0.15-0.42)	40.6 (40.1-41.0)	26 (23-29)	1:320 (1:320-1.320)	423 (256-589)	0
IV	0 (controls)	1×10^{7}	3/3	5.7 (5-6)	0.03 (0.008-0.06)	40.5 (40.3-40.7)	27 (24-32)	1:201 (1:160-1:320)	798 (643-911)	0
IV	0 (controls)	1×10^{4}	0/3	NONE	NONE	39.7 (39.2-40.0)	27 (24-28)	Neg. (Neg.-Neg.)	863 (643-1036)	0
I	22	1×10^{9}	2/2	7.5 (7-8)	0.13 (0.09-0.18)	39.5 (39.5-39.5)	28 (26-29)	1.227 (1:160-1:320)	840 (840-840)	0
IV	24	1×10^{10}	4/4	6.5 (6-8)	0.11 (0.008-0.40)	40.3 (39.5-40.7)	26 (22-30)	1:135 (1.80-1.320)	661 (500-786)	0
I	26	1×10^{9}	2/2	9.0 (8-10)	0.04 (0.005-0.08)	41.0 (40.9-41.1)	30 (28-32)	1:113 (1:80-1:160)	572 (536-607)	0
I	30	1×10^{9}	2/2	10.0 (10-10)	0.08 (0.005-0.15)	40.0 (39.3-40.7)	32 (30-33)	1:113 (1.80-1:160)	786 (786-786)	0
II + III	36	1×10^{9}	4/4	7.5 (6-10)	0.02 (0.01-0.04)	39.8 (39.6-40.1)	25 (21-28)	1:33 (Neg.-1 320)	742 (476-857)	0
IV	36	1×10^{10}	1/4	13.0 (13-13)	0.002 (0.002-0.002)	40.1 (39.7-40.3)	30 (26-33)	1:13 (Neg.-1:80)	790 (536-1000)	0
II + III	42	1×10^{9}	4/4	8.0 (7-10)	0.01 (0.003-0.02)	39.7 (39.5-40.2)	29 (28-31)	1.37 (Neg.-1:320)	576 (238-893)	0
IV	48	1×10^{10}	0/4	NONE	NONE	40.1 (39.6-41.0)	31 (29-34)	1:2 (Neg.-1:5)	875 (661-982)	0
IV	60	1×10^{10}	0/4	NONE	NONE	40.6 (40.0-40.9)	30 (28-31)	1:3 (Neg.-1:20)	857 (786-1018)	0

[a] Characteristics of reactions based on data collected for 28 days post inoculation.

[b] Detected by either thick blood films or subinoculations.

[c] Range of means.

B. bigemina parasitized erythrocytes were 2 days. The average maximum parasitemias were 0.29, 0.13, 0.04, and 0.08% for calves infected with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 0, 22, 26, and 30 kRad, respectively. The average maximum morning rectal temperatures were 40.6, 39.5, 41.0, and 40.0°C for calves infected with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 0, 22, 26, and 30 kRad, respectively. The average minimum packed cell volumes were 26, 28, 30, and 32% for calves infected with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 0, 22, 26, and 30 kRad, respectively. The average maximum CF titers were 1:320, 1:227, 1:113, and 1:113 during 28 days following inoculation with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 0, 22, 26, and 30 kRad, respectively. The average daily gains were 423, 840, 572, and 786 g/day for 28 days following inoculation with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 0, 22, 26, and 30 kRad, respectively. The average prepatent periods, parasitemias, maximum temperatures, minimum packed cell volumes, average daily gains, and immune responses were similar in calves infected with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 0, 22, 26, and 30 kRad following challenge with 1 x 10^{10} nonirradiated B. bigemina.

One calf inoculated with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 30 kRad died 34 days after inoculation with chronic bacterial pneumonia. One calf, 7 weeks after being inoculated with blood containing 1 x 10^9 B. bigemina parasitized

erythrocytes exposed to 26 kRad, developed a severe systemic reaction following inoculation of blood containing 1 x 10^{10} non-irradiated B. bigemina parasitized erythrocytes. Within 2 minutes of completion of the injection, the calf suffered intense respiratory distress, collapsed on its side and showed nystagmus. Coughing produced a cream colored frothy fluid which was mixed with blood. The calf breathed more easily after 5 minutes and stood up after 15 minutes. Its posture was characteristic of dyspneic animals with the neck extended, head lowered, mouth opened, and tongue protruded. Abdominal respiratory movements were marked and grunting accompanied expirations.

Experiments II and III

The second experiment was undertaken to determine the effect of gamma radiation dosages of 36 and 42 kRad on the infectivity and immunogenicity of B. bigemina in calves given 2 inoculations at 14 day intervals. The results of experiment II summarized in Figures 9-12 and Table 2 (p. 71) show that calves did not develop progressive parasitemias following the first inoculation of 1 x 10^9 B. bigemina parasitized erythrocytes exposed to dosages of 36 and 42 kRad. The same calves, however, developed progressive parasitemias following the second inoculation of 1 x 10^9 B. bigemina parasitized erythrocytes exposed to dosages of 36 and 42 kRad. The average prepatent periods were 8 and 9 days for calves infected with 1 x 10^9 B. bigemina parasitized erythrocytes

Figure 9. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 2 calves inoculated (I) twice with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 36 kRad and challenged (C) 3 weeks after the last inoculation with 1 x 10^{10} B. bigemina parasitized erythrocytes. One calf (*) died with severe systemic reactions following challenge with blood containing 1 x 10^{10} B. bigemina parasitized erythrocytes.

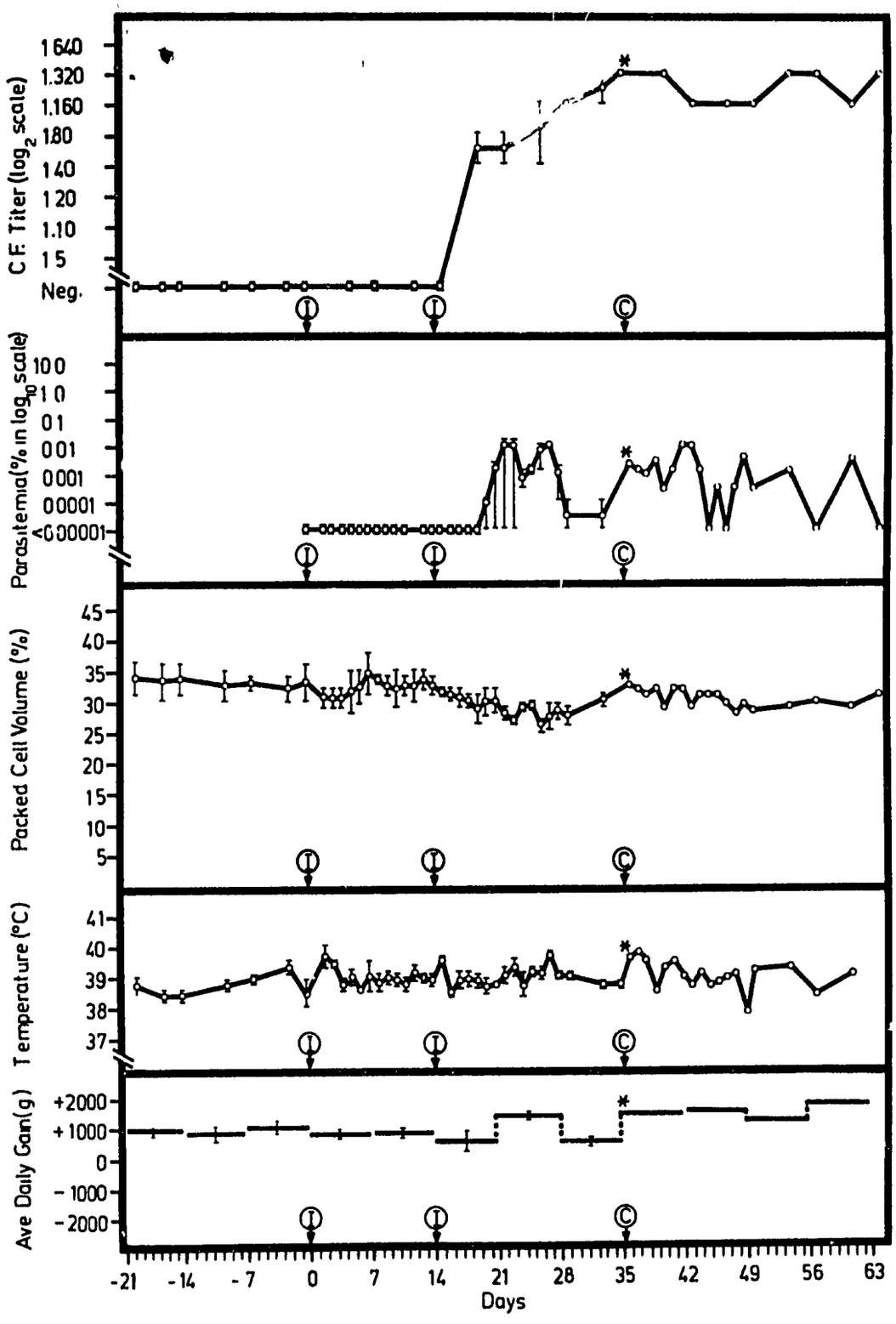

C.F. Titer (log2 scale)
1640
1.320
1.160
1.80
140
120
1.10
15
Neg.
Parasitemia (% in log10 scale)
100
10
01
001
0001
00001
<00001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave Daily Gain (g)
+2000
+1000
0
-1000
-2000
I
I
C
-21
-14
-7
0
7
14
21
28
35
42
49
56
63
Days

Figure 10. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures, and average daily gains for 2 calves inoculated (I) twice with 1×10^9 B. bigemina parasitized erythrocytes exposed to 42 kRad, and challenged (C) 3 weeks after the last inoculation with 1×10^{10} B. bigemina parasitized erythrocytes.

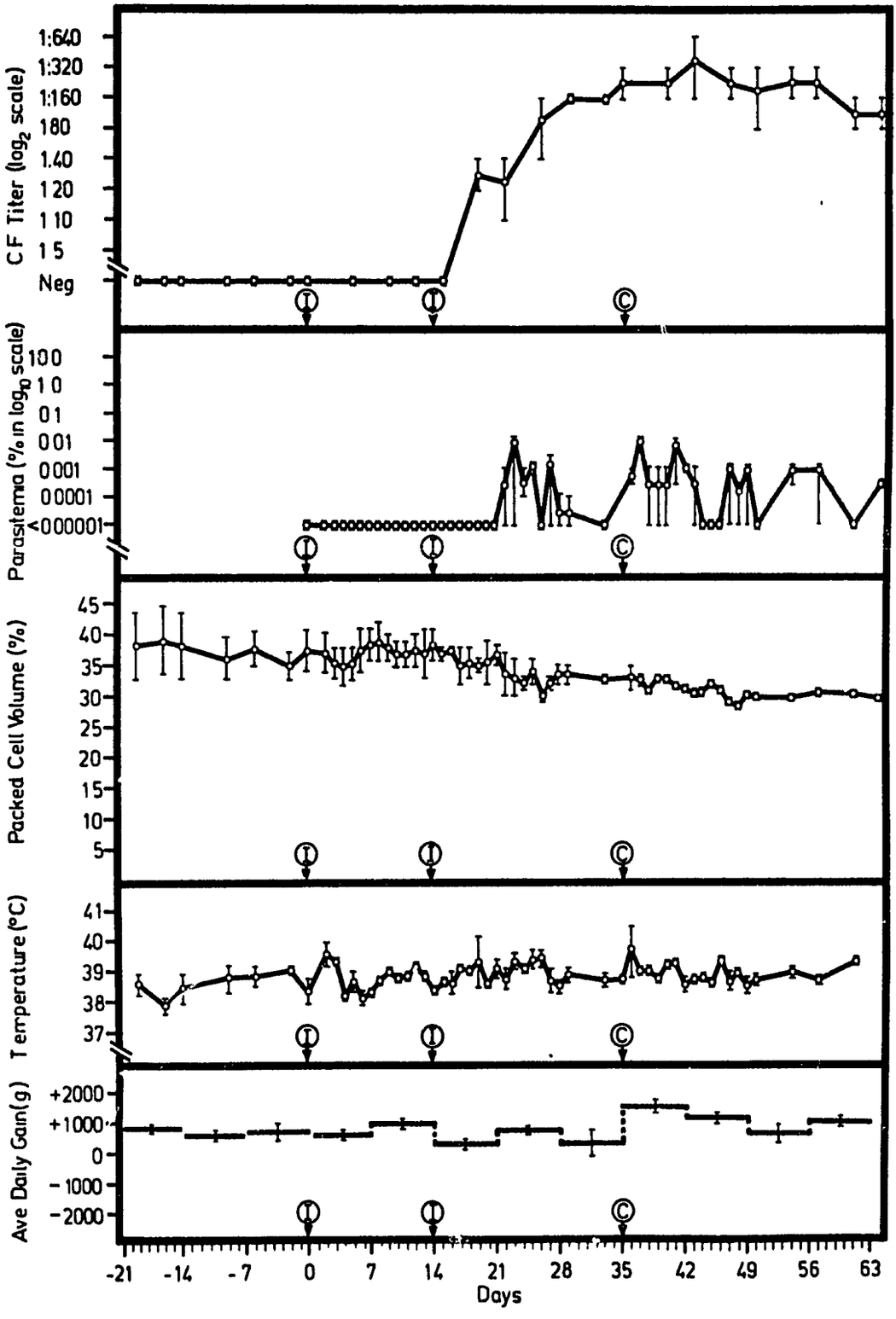

CF Titer (log2 scale)
1:640
1:320
1:160
180
1.40
120
110
15
Neg
Parasitemia (% in log10 scale)
100
10
01
001
0001
00001
<000001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave Daily Gain (g)
+2000
+1000
0
-1000
-2000
I
I
C
-21
-14
-7
0
7
14
21
28
35
42
49
56
63
Days

Figure 11. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 1 calf inoculated (I) with 1 x 10^9 nonirradiated B. bigemina parasitized erythrocytes, and challenged (C) 5 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes.

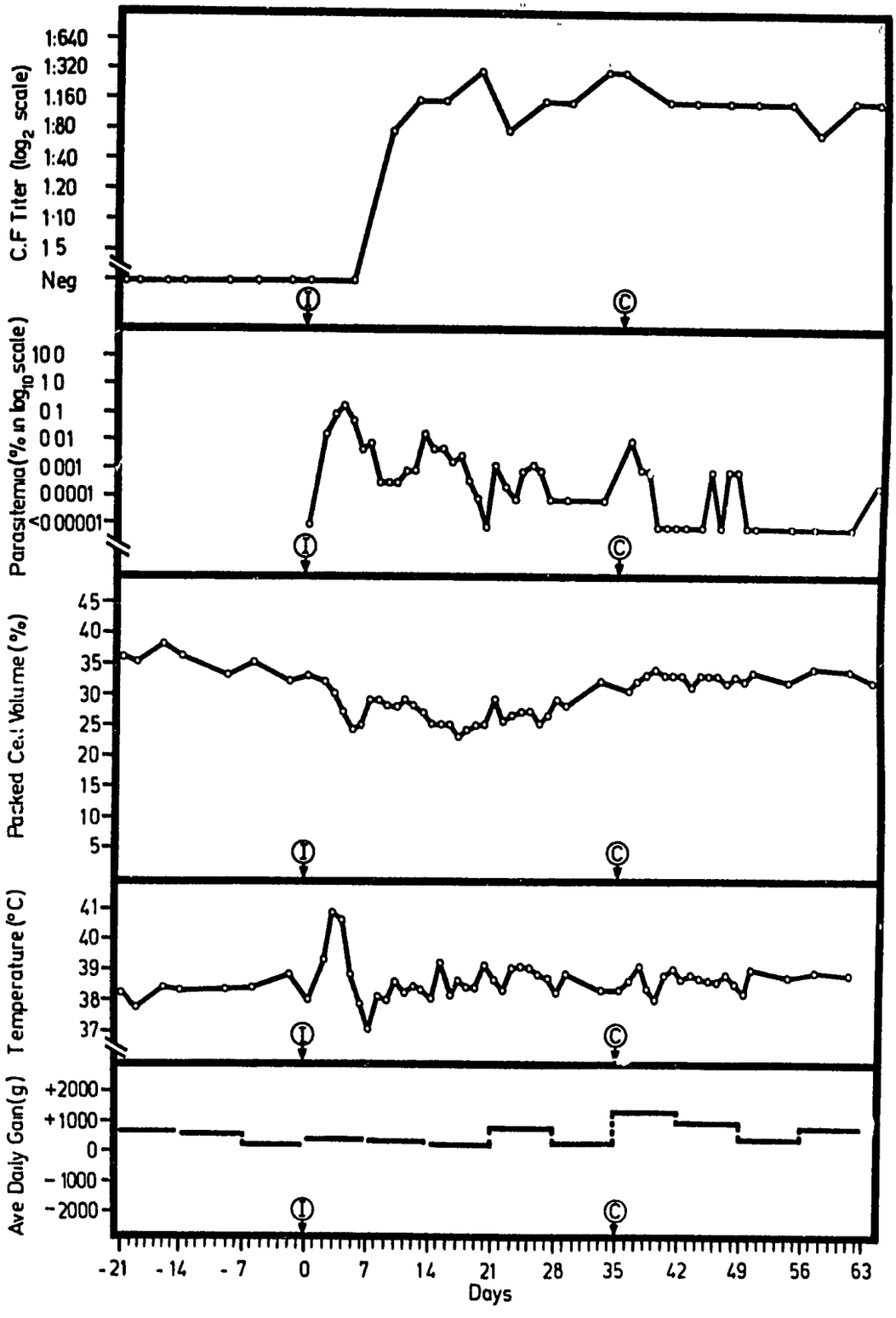
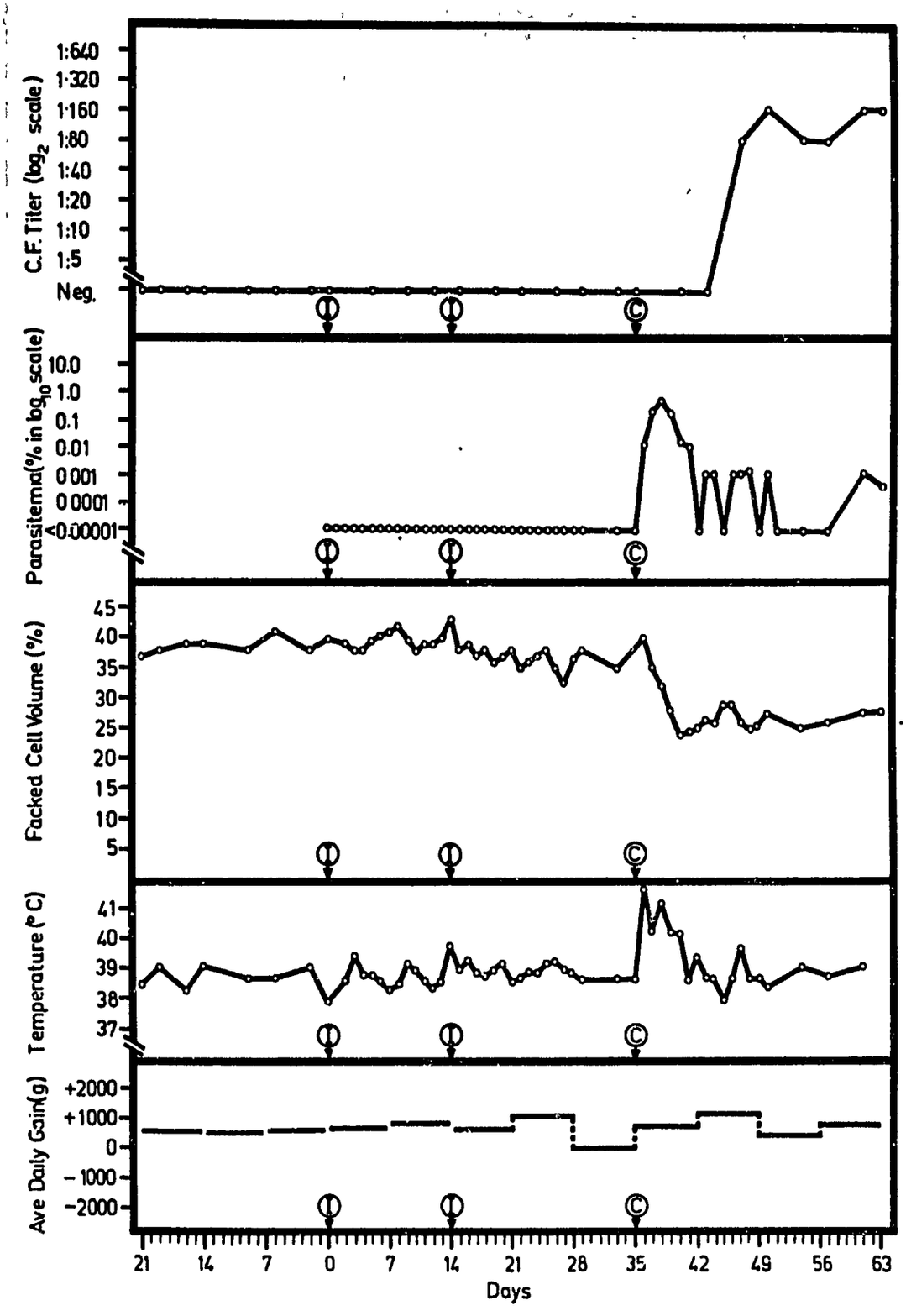

C.F Titer (log2 scale)
1:640
1:320
1.160
1:80
1:40
1.20
1·10
1 5
Neg
Parasitemia (% in log10 scale)
100
10
01
001
0001
00001
<0 00001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave Daily Gain (g)
+2000
+1000
0
-1000
-2000
I
C
-21
-14
-7
0
7
14
21
28
35
42
49
56
63
Days

Figure 12. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 1 calf inoculated (I) twice with 12 ml normal blood irradiated with 42 kRad, and challenged (C) 3 weeks after the last inoculation with 1×10^{10} B. bigemina parasitized erythrocytes.


C.F. Titer (log_2 scale)
1:640
1:320
1:160
1:80
1:40
1:20
1:10
1:5
Neg.
Parasitemia (% in log_{10} scale)
10.0
1.0
0.1
0.01
0.001
0.0001
<0.00001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave Daily Gain (g)
+2000
+1000
0
-1000
-2000
21
14
7
0
7
14
21
28
35
42
49
56
63
Days

exposed to 36 and 42 kRad, respectively. The average prepatent periods for calves infected with nonirradiated B. bigemina parasitized erythrocytes were 2 days.

The third experiment was undertaken to determine the effect of gamma radiation dosages of 36 and 42 kRad on the infectivity and immunogenicity of B. bigemina in calves given one inoculation.

The results of experiment III summarized in Figures 13 and 14 and Table 2 (p. 71) show that calves infected with 1×10^9 B. bigemina parasitized erythrocytes exposed to dosages of 36 and 42 kRad developed progressive parasitemias which were delayed in comparison to calves inoculated with nonirradiated B. bigemina. The prepatent period was 7 days for all calves infected with 1×10^9 B. bigemina parasitized erythrocytes exposed to 36 and 42 kRad. The average prepatent period for calves infected with nonirradiated B. bigemina parasitized erythrocytes was 2 days.

The average maximum parasitemias were 0.29, 0.02, and 0.01% for calves in experiments II and III infected with 1×10^9 B. bigemina parasitized erythrocytes exposed to 0, 36, and 42 kRad, respectively. The average maximum morning rectal temperatures were 40.6, 39.8, and 39.7°C for calves in experiments II and III inoculated with 1×10^9 B. bigemina parasitized erythrocytes exposed to 0, 36, and 42 kRad, respectively. The average minimum packed cell volumes were 26, 25, and 29% for calves in experiments II and III inoculated with 1×10^9 B. bigemina parasitized erythrocytes exposed to 0, 36, and 42 kRad, respectively. The average

Figure 13. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 2 calves inoculated (I) with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 36 kRad, and challenged (C) 3 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes.

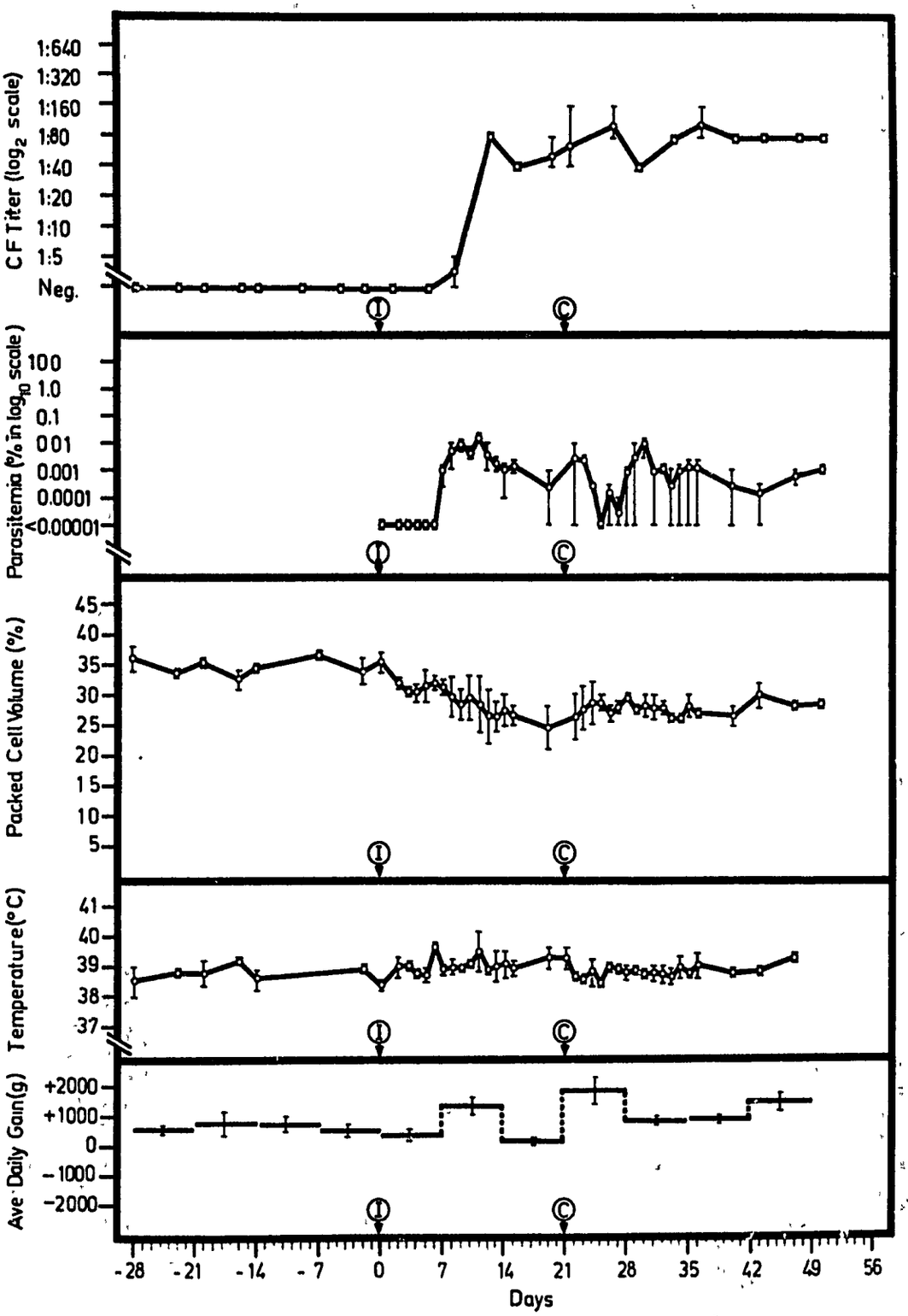
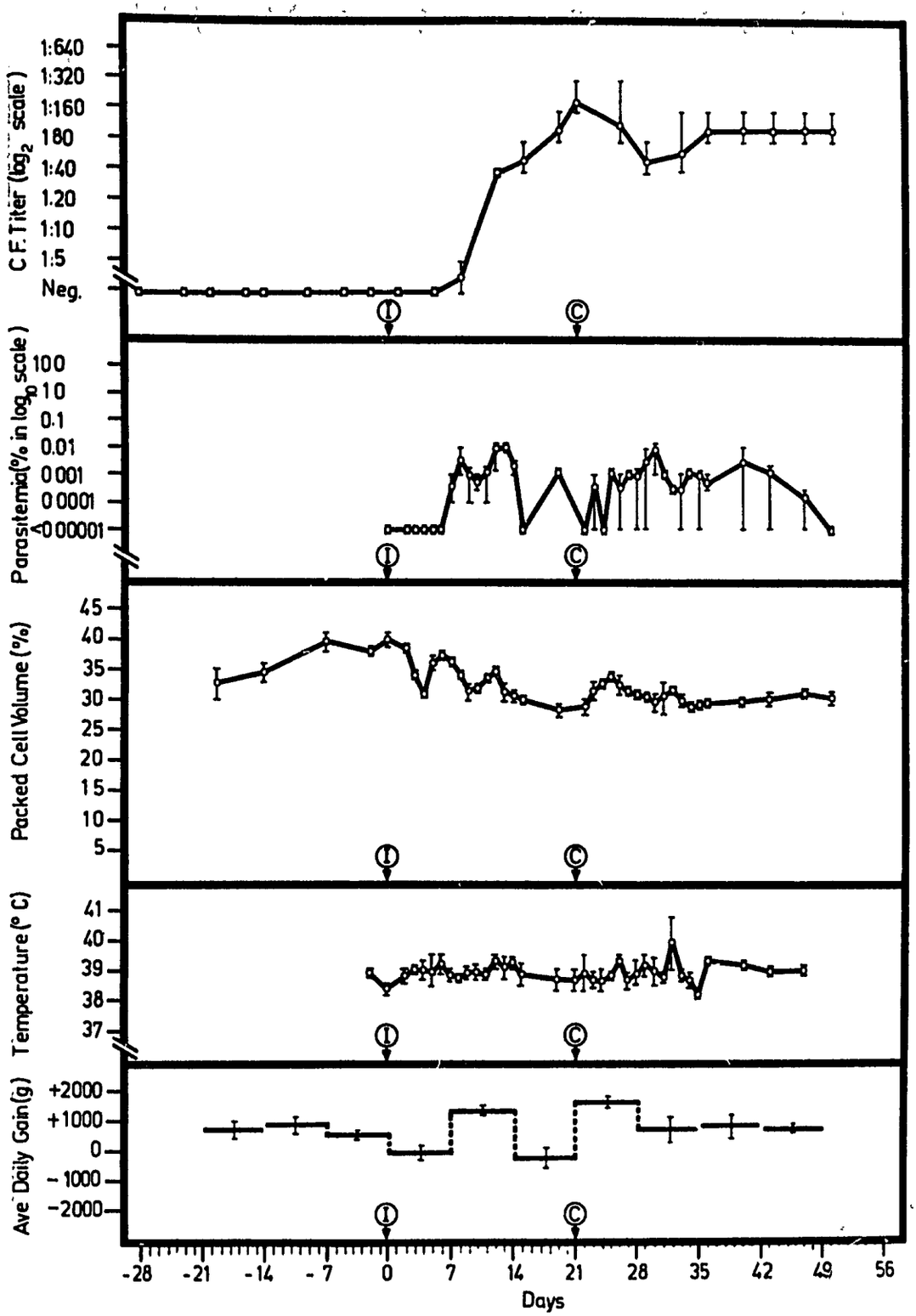

C F Titer (log2 scale)
1:640
1:320
1:160
1:80
1:40
1:20
1:10
1:5
Neg.
Parasitemia (% in log10 scale)
100
1.0
0.1
001
0.001
0.0001
<0.00001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature(°C)
41
40
39
38
37
Ave Daily Gain(g)
+2000
+1000
0
-1000
-2000
I
C
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days

Figure 14. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 2 calves inoculated (I) with 1×10^9 B. bigemina parasitized erythrocytes exposed to 42 kRad, and challenged (C) 3 weeks after the last inoculation with 1×10^{10} B. bigemina parasitized erythrocytes.


C.F. Titer (log_2 scale)
1:640
1:320
1:160
180
1:40
1.20
1:10
1:5
Neg.
Parasitemia (% in log_{10} scale)
100
10
0.1
0.01
0 001
0 0001
<0 00001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (° C)
41
40
39
38
37
Ave Daily Gain (g)
+2000
+1000
0
- 1000
-2000
I
C
-28
-21
-14
- 7
0
7
14
21
28
35
42
49
56
Days

maximum CF titers were 1:320, 1:33, and 1:37 following inoculation with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 0, 36, and 42 kRad, respectively. The average daily gains were 423, 742, and 576 g/day for 28 days following inoculation with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 0, 36, and 42 kRad, respectively. The average prepatent periods, parasitemias, maximum temperatures, minimum packed cell volumes, average daily gains and immune responses were similar in calves infected with 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 0, 36, and 42 kRad following challenge with 1 x 10^{10} nonirradiated B. bigemina.

Two calves in experiment II, 21 days after being inoculated the second time with blood containing 1 x 10^9 B. bigemina parasitized erythrocytes exposed to 36 kRad developed severe systemic reactions. Within 2 minutes of completion of the injections, the calves suffered intense respiratory distress. One calf collapsed on its side and showed nystagmus. Coughing produced a cream colored frothy fluid which was mixed with blood and respiration ceased 10 minutes after injection. Post-mortem examination of the calf revealed lesions which were confined largely to the respiratory system. There were severe intra-alveolar and interstitial edema and emphysema with intra-alveolar hemorrhage. The trachea and major bronchi contained a cream colored frothy fluid which was bloodstained. The second calf breathed more easily after 5 minutes and stood up after 15 minutes. Its posture was

characteristic of dyspneic animals with the neck extended, head lowered, mouth opened, and tongue protruded. Abdominal respiratory movements were marked and grunting accompanied expirations.

Experiments IV and V

The fourth experiment was undertaken to determine the effect of gamma radiation dosages of 24, 36, 48, and 60 kRad on the infectivity and immunogenicity of B. bigemina in calves.

The results of experiment IV summarized in Figures 15-23 and Tables 2 (p. 71) and 3 show that calves inoculated with B. bigemina parasitized blood exposed to 24 kRad developed progressive parasitemias which were delayed in comparison to calves inoculated with 1×10^{10} nonirradiated B. bigemina. Three out of 4 calves receiving parasitized blood irradiated at 36 kRad did not develop progressive parasitemias. Progressive infections were prevented by exposure to irradiation at 48 and 60 kRad. The average prepatent periods were 6.5 and 13 days for calves infected with 1×10^{10} B. bigemina parasitized erythrocytes exposed to 24 and 36 kRad, respectively. The average prepatent period for calves infected with 1×10^{10} nonirradiated B. bigemina parasitized erythrocytes was 2 days. The average prepatent period for calves infected with 1×10^{7} nonirradiated B. bigemina parasitized erythrocytes was 5.7 days. Calves inoculated with 1×10^{4} nonirradiated B. bigemina parasitized erythrocytes did not develop progressive parasitemias.

Figure 15. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 4 calves inoculated (I) with 1×10^{10} B. bigemina parasitized erythrocytes exposed to 24 kRad, and challenged (C) 4 weeks later with 1×10^{10} B. bigemina parasitized erythrocytes.

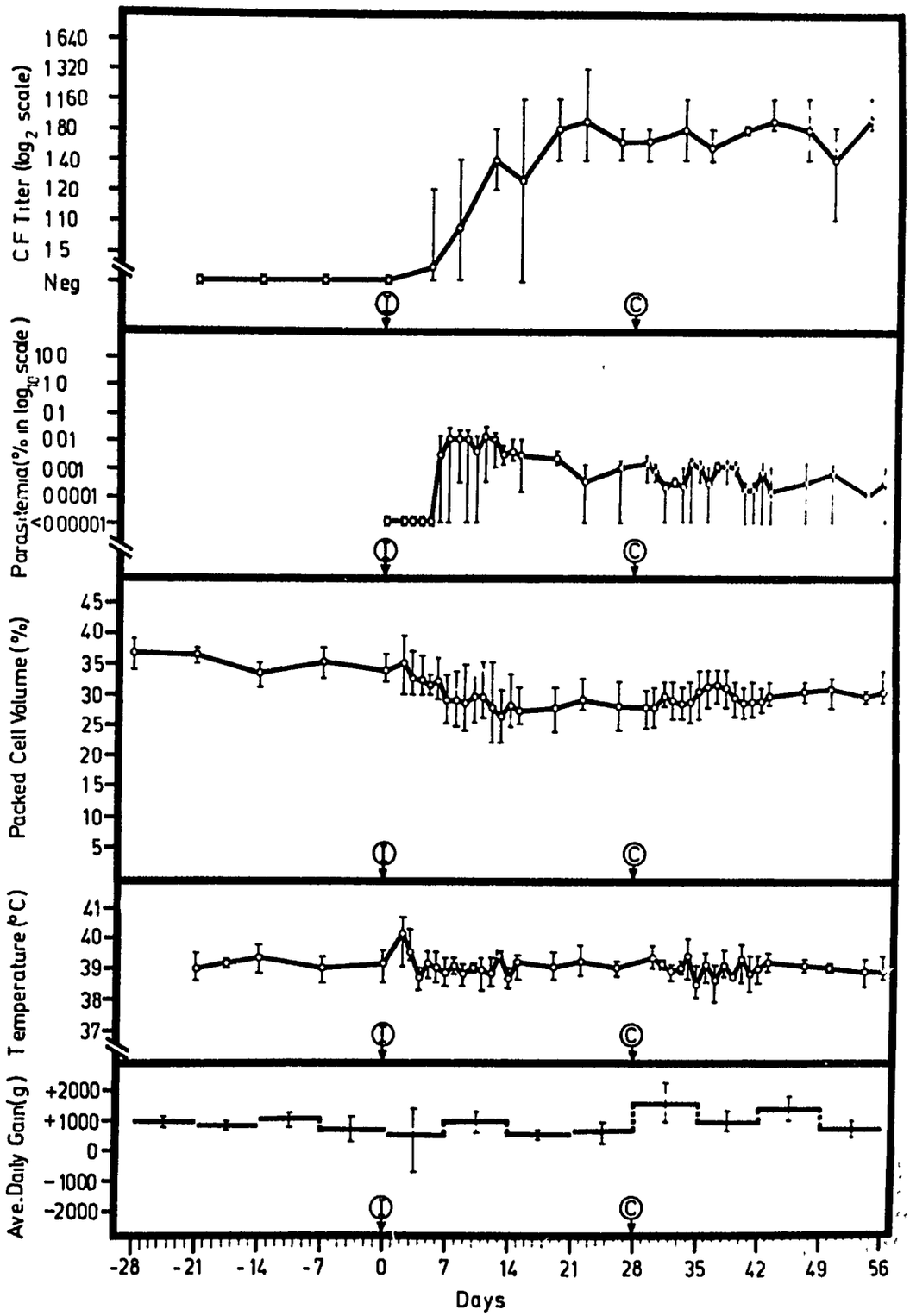

CF Titer (log2 scale)
1640
1320
1160
180
140
120
110
15
Neg
Parasitemia (% in log10 scale)
100
10
01
001
0001
00001
<00000
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave. Daily Gain (g)
+2000
+1000
0
-1000
-2000
I
C
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days

Figure 16. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 4 calves inoculated (I) with 1 x 10^{10} B. bigemina parasitized erythrocytes exposed to 36 kRad, and challenged (C) 4 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes. One calf died (*) with severe systemic reactions following challenge with blood containing 1 x 10^{10} B. bigemina parasitized erythrocytes.

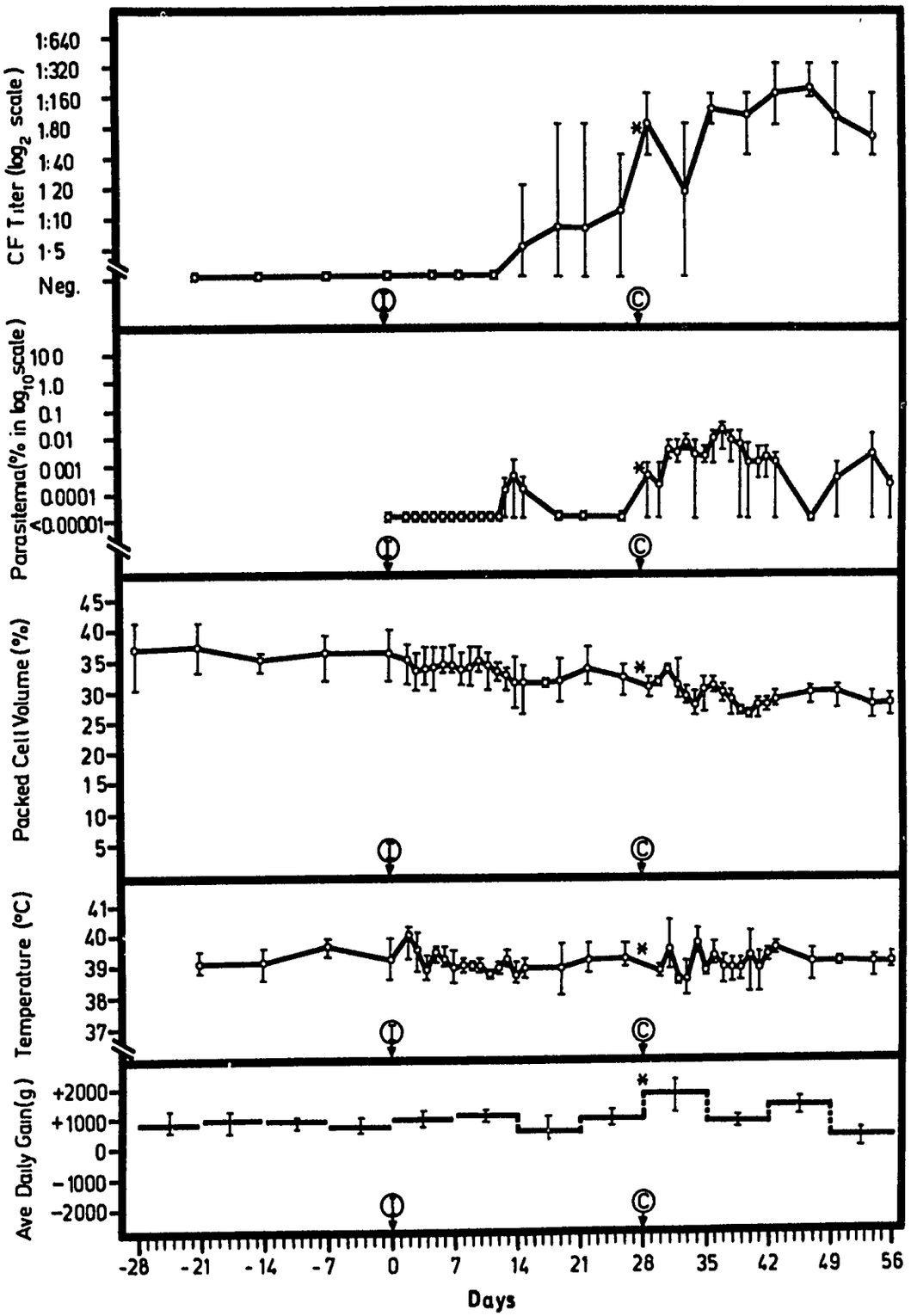

CF Titer (log2 scale)
1:640
1:320
1:160
1.80
1:40
1 20
1:10
1·5
Neg.
Parasitemia(% in log10 scale)
100
1.0
0.1
0.01
0 001
0.0001
<0.00001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave Daily Gain(g)
+2000
+1000
0
-1000
-2000
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days

Figure 17. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 4 calves inoculated (I) with 1 x 10^{10} B. bigemina parasitized erythrocytes exposed to 48 kRad, and challenged (C) 4 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes. The 4 calves were negative (N) on subinoculation the day of challenge.

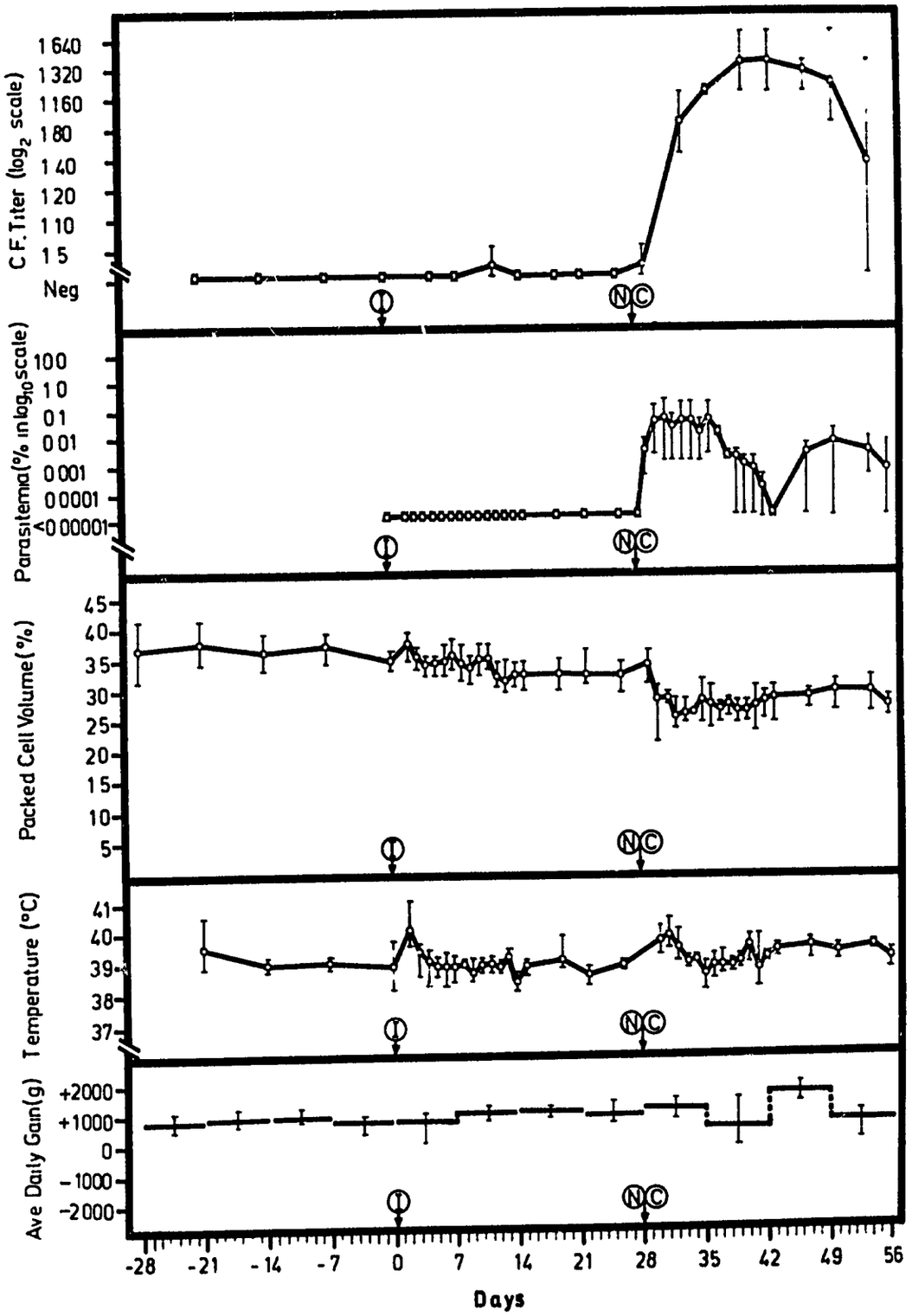

C.F. Titer (log2 scale)
1 640
1 320
1 160
1 80
1 40
1 20
1 10
1 5
Neg
Parasitemia (% in log10 scale)
100
10
01
001
0001
00001
<0 00001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave Daily Gain (g)
+2000
+1000
0
-1000
-2000
Φ
NC
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days

Figure 18. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 4 calves inoculated (I) with 1 x 10^{10} B. bigemina parasitized erythrocytes exposed to 60 kRad and challenged (C) 4 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes. The 4 calves were negative (N) on subinoculation the day of challenge.

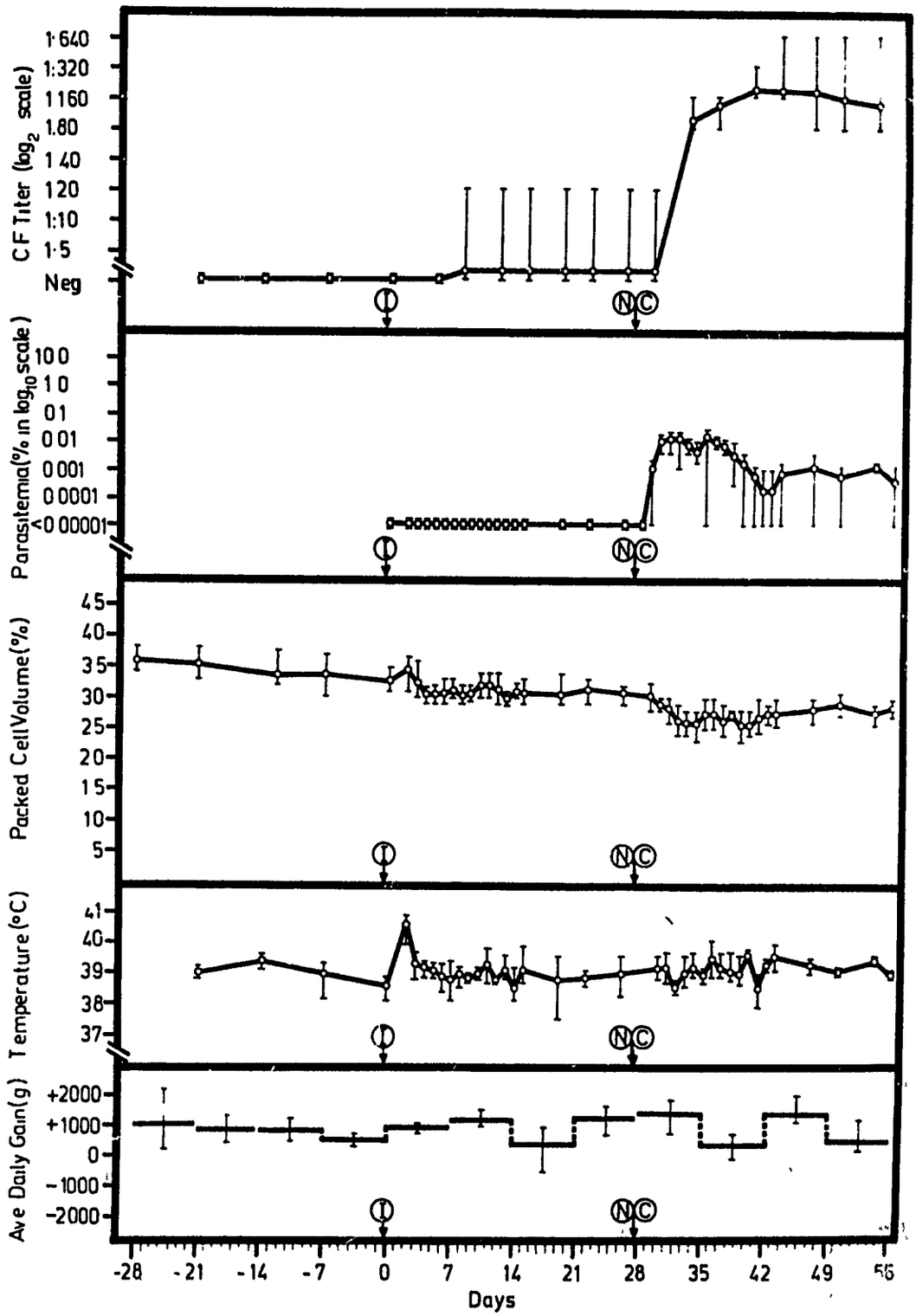

CF Titer (log2 scale)
1:640
1:320
1160
1.80
1 40
120
1:10
1·5
Neg
Parasitemia(% in log10 scale)
100
10
01
001
0001
00001
<0 00001
Packed Cell Volume(%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave Daily Gain(g)
+2000
+1000
0
-1000
-2000
I
N C
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days

Figure 19. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 3 calves inoculated (I) with 1 x 10^{10} nonirradiated B. bigemina parasitized erythrocytes and challenged (C) 4 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes.

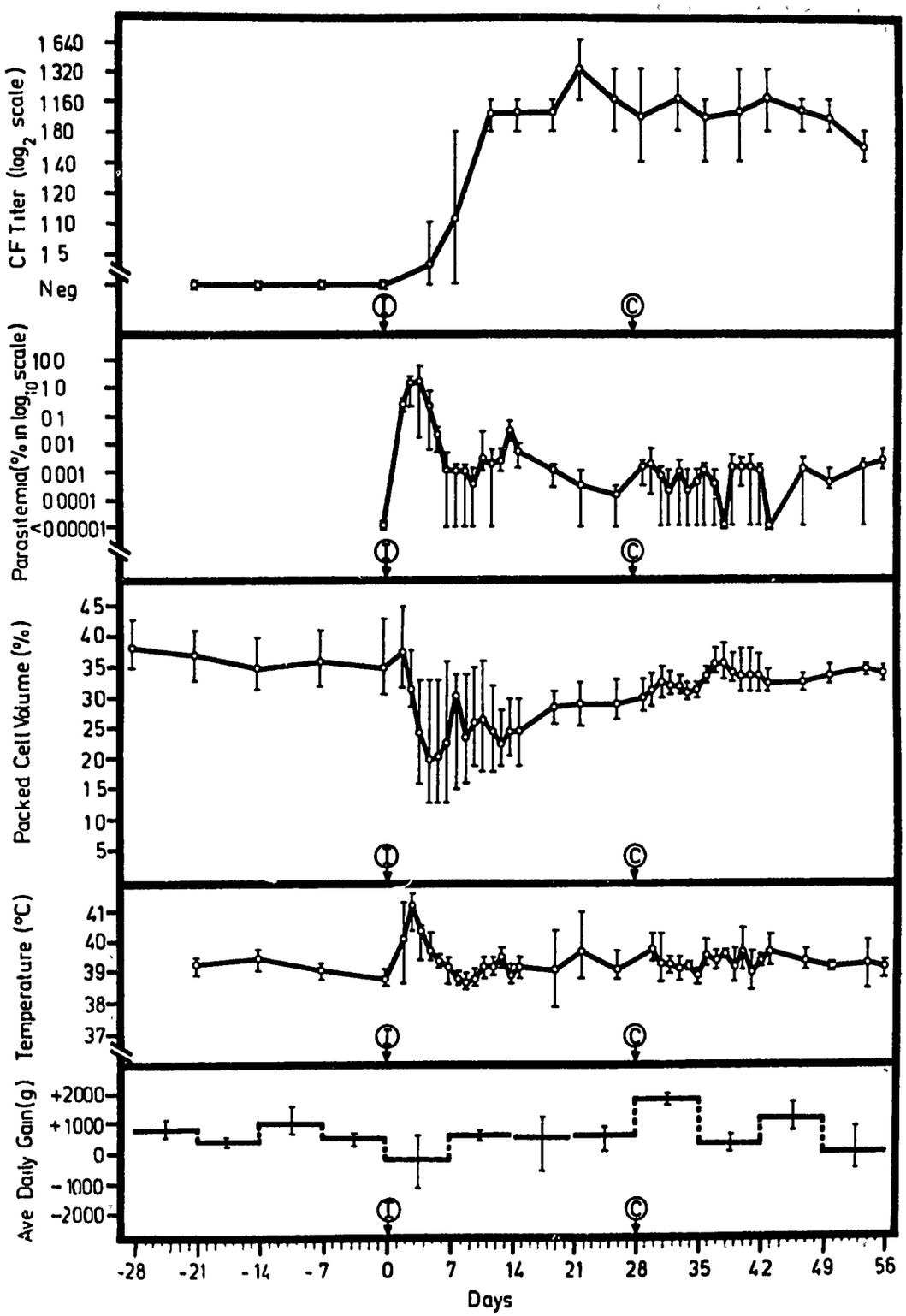

CF Titer (log2 scale)
1 640
1 320
1 160
1 80
1 40
1 20
1 10
1 5
Neg
Parasitemia(% in log10 scale)
100
10
01
001
0001
00001
<000001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave Daily Gain(g)
+2000
+1000
0
-1000
-2000
I
C
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days

Figure 20. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 3 calves inoculated (I) with 1×10^7 nonirradiated B. bigemina parasitized erythrocytes, and challenged (C) 4 weeks later with 1×10^{10} B. bigemina parasitized erythrocytes.

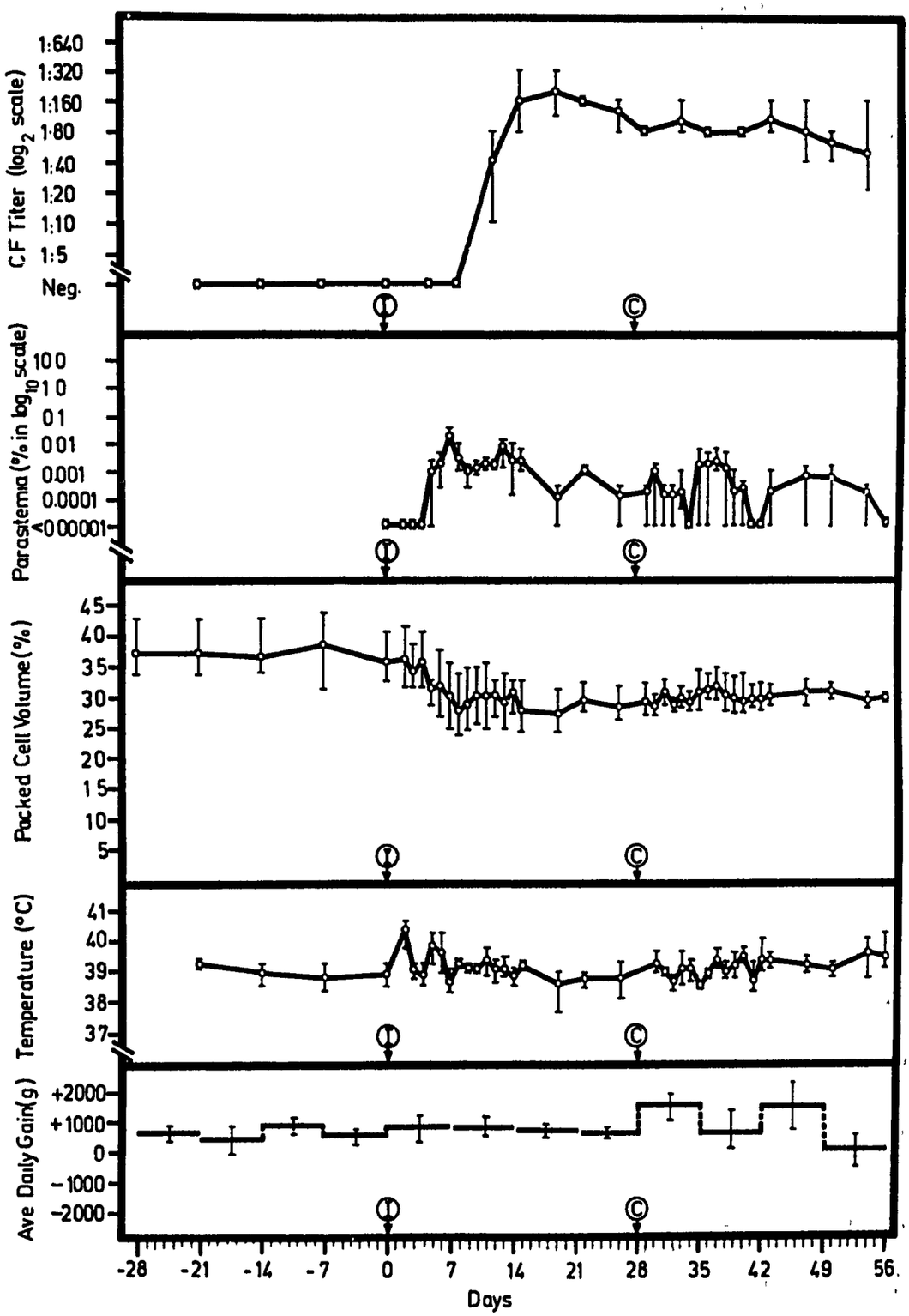

CF Titer (log2 scale)
1:640
1:320
1:160
1:80
1:40
1:20
1:10
1:5
Neg.
Parasitemia (% in log10 scale)
100
10
01
001
0.001
0.0001
<0.00001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave Daily Gain (g)
+2000
+1000
0
-1000
-2000
I
C
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days

Figure 21. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 3 calves inoculated (I) with 1 x 10^4 nonirradiated B. bigemina parasitized erythrocytes and challenged (C) 4 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes. One calf died (*) with severe systemic reactions following challenge with blood containing 1 x 10^{10} B. bigemina parasitized erythrocytes.

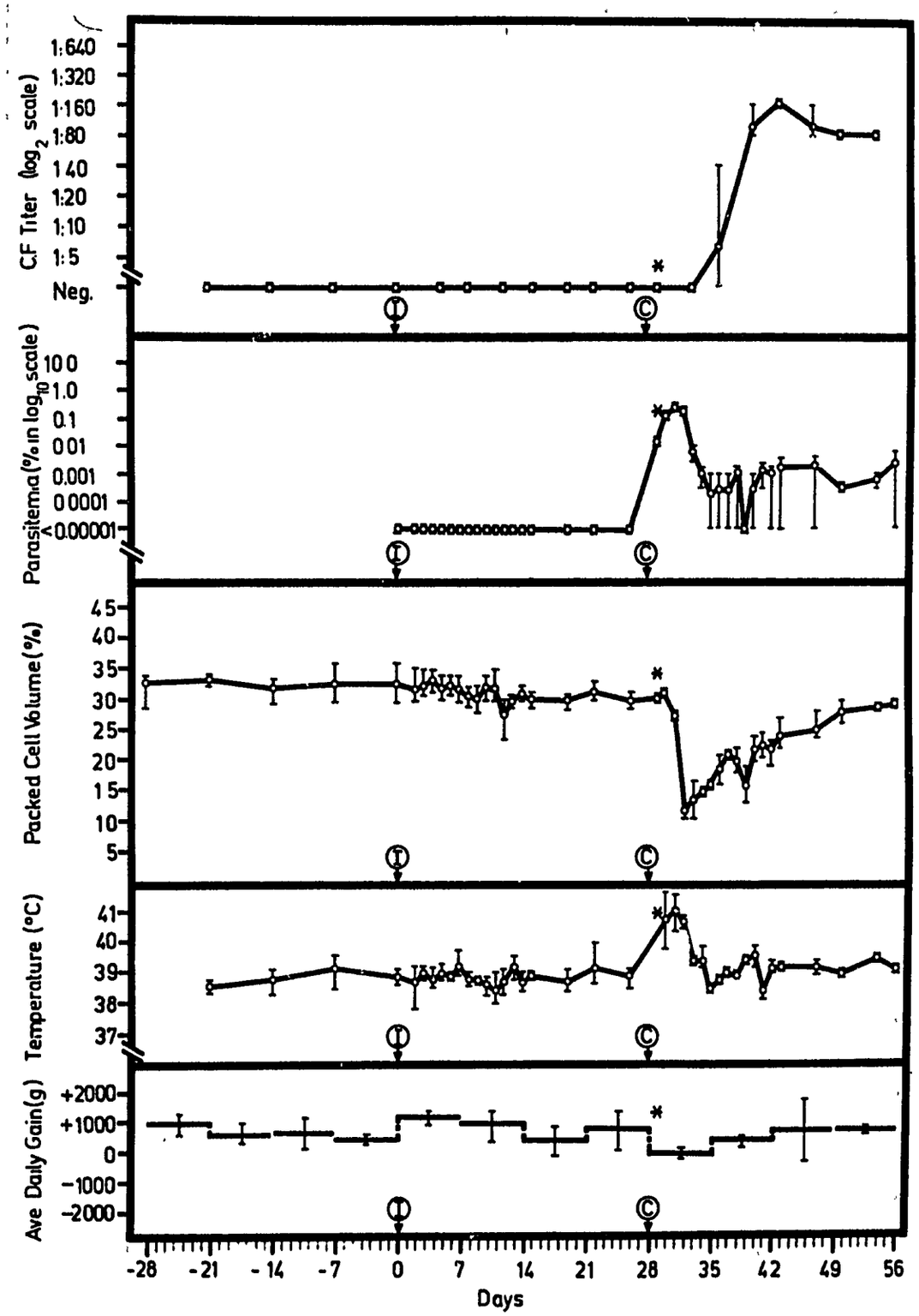

CF Titer (log2 scale)
1:640
1:320
1:160
1:80
1:40
1:20
1:10
1:5
Neg.
Parasitemia (% in log10 scale)
10.0
1.0
0.1
0.01
0.001
0.0001
<0.00001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave Daily Gain (g)
+2000
+1000
0
−1000
−2000
T
C
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days

Figure 22. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 3 calves inoculated (I) with 1 x 10^{10} heat inactivated B. bigemina parasitized erythrocytes and challenged (C) 4 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes. The 3 calves were negative (N) on subinoculation the day of challenge.

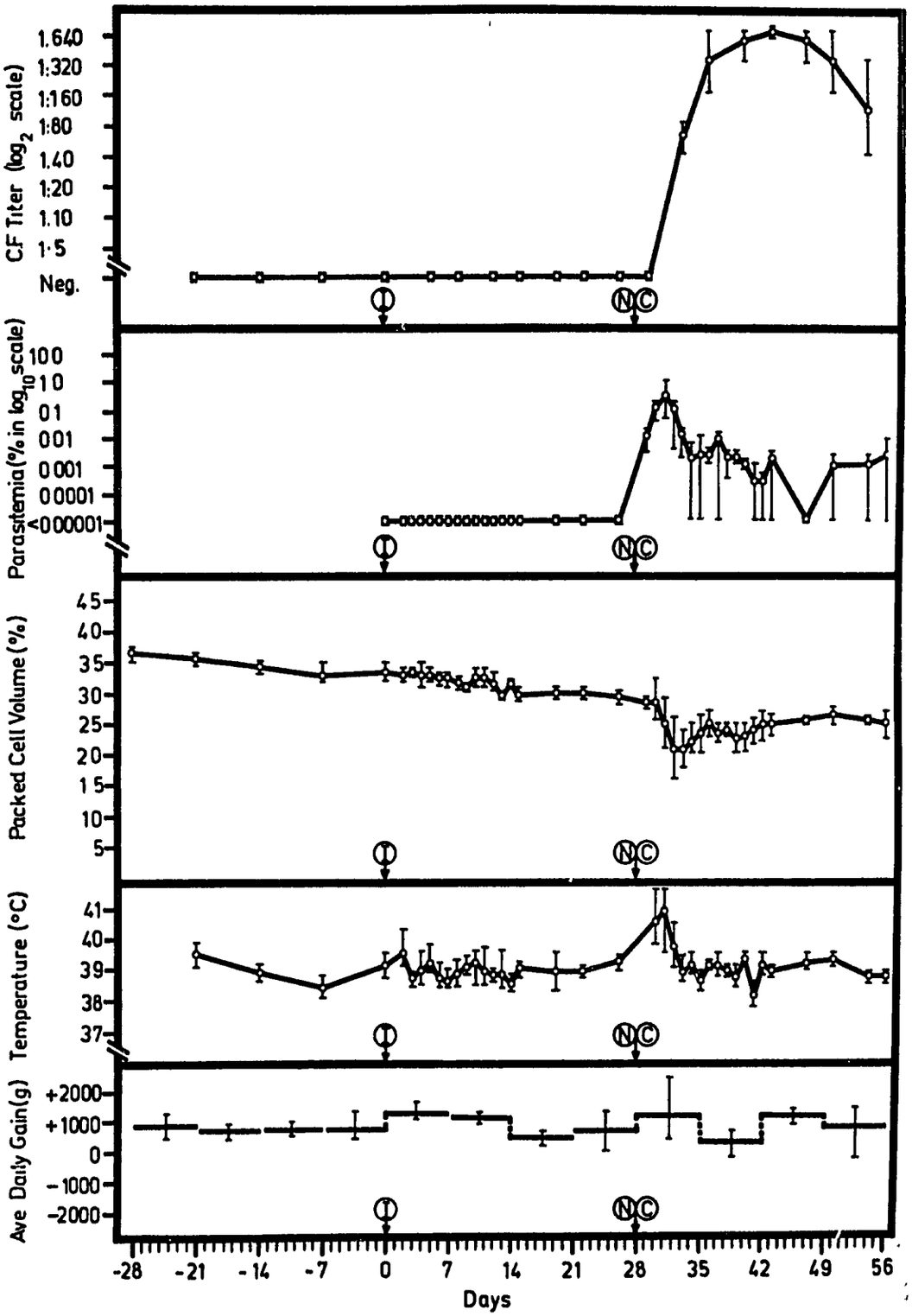

CF Titer (log2 scale)
1.640
1:320
1:160
1:80
1.40
1:20
1.10
1·5
Neg.
Parasitemia (% in log10 scale)
100
10
01
001
0001
00001
<000001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave Daily Gain (g)
+2000
+1000
0
-1000
-2000
P
N
C
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days

Figure 23. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 3 calves inoculated (I) with 10 ml normal blood irradiated with 36 kRad, and challenged (C) 4 weeks later with 1×10^{10} B. bigemina parasitized erythrocytes. One calf died (*) with acute babesiosis following challenge with 1×10^{10} B. bigemina parasitized erythrocytes.

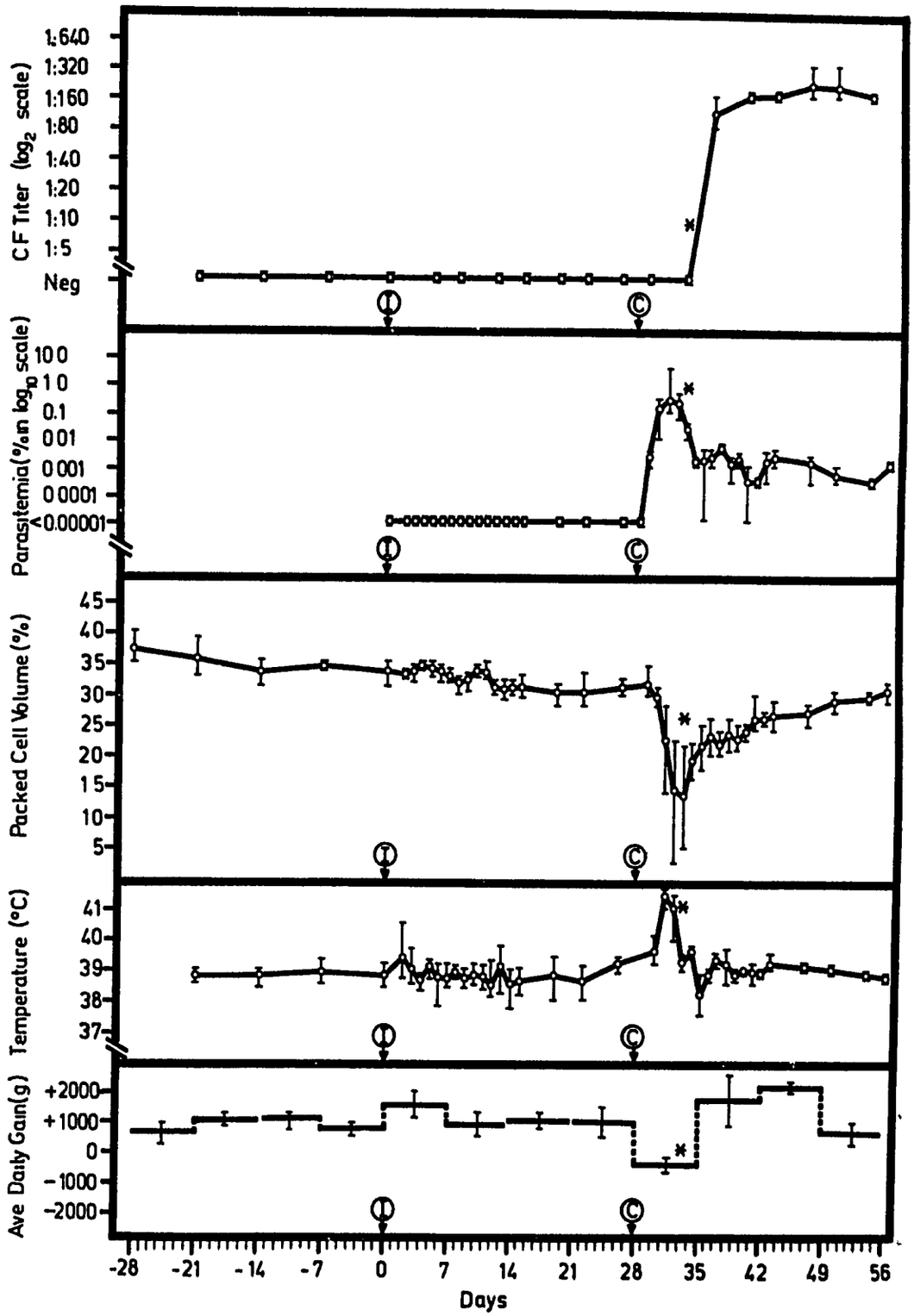

CF Titer (log2 scale)
1:640
1:320
1:160
1:80
1:40
1:20
1:10
1:5
Neg
Parasitemia (% in log10 scale)
100
10
0.1
0.01
0.001
0.0001
<0.00001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave Daily Gain (g)
+2000
+1000
0
-1000
-2000
I
C
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days

Table 3. Immune Response of Calves Inoculated Once with Irradiated *Babesia bigemina* and Challenged 4 Weeks Later with Nonirradiated *B. bigemina*. Summary of Experiments IV and V.

Exp. No.	Treatment of Inoculum	Size of Inoculum	Reaction After Challenge with 1×10^{10} Unirradiated *B. bigemina* Parasitized Erythrocytes[a]							
			No. Calves with Parasitemia / No. Calves Challenged	Prepatent Period in Days	Maximum % Parasitized Erythrocytes	Maximum Temperature in °C	Minimum Packed Cell Volume in %	Maximum Complement Fixation Titer	Average Daily Gain in g/day	Deaths
IV	0 kRad (Control)	1×10^{10} *B. bigemina*	3/3	1.0 (1-1)[b]	0.006 (0.001-0.008)	40.2 (39.9-40.5)	29 (28-31)	1:201 (1:80-1:320)	887 (786-964)	0
IV	0 kRad (Control)	1×10^{7} *B. bigemina*	3/3	1.7 (1-3)	0.004 (0.002-0.008)	40.2 (40.1-40.3)	28 (28-30)	1:127 (1:80-1:160)	976 (893-1036)	0
IV	0 kRad (Control)	1×10^{4} *B. bigemina*	3/3	1.0 (1-1)	0.5 (0.5-0.5)	41.3 (40.9-41.7)	11 (10-12)	1:160 (1:160-1:160)	813 (696-929)	1[c]
IV	Heat Inact. (Control)	1×10^{10} *B. bigemina*	3/3	1.0 (1-1)	0.8 (0.4-1.0)	41.1 (39.8-41.8)	19 (16-22)	1:640 (1:640-1:640)	815 (482-1107)	0
IV	36 kRad (Control)	10 ml Normal Blood	3/3	1.0 (1-1)	0.6 (0.1-1.0)	41.4 (41.0-41.7)	10 (3-14)	1:227 (1:160-1:320)	982 (786-1179)	1[d]
IV	24 kRad	1×10^{10} *B. bigemina*	4/4	1.0 (1-1)	0.003 (0.002-0.005)	39.7 (39.6-40.0)	27 (25-30)	1:134 (1:80-1:160)	1093 (1000-1264)	0
IV	36 kRad	1×10^{10} *B. bigemina*	4/4	2.0 (1-4)	0.02 (0.008-0.04)	40.0 (39.4-40.5)	26 (26-27)	1:201 (1:160-1:320)	1060 (839-1214)	1[c]
IV	48 kRad	1×10^{10} *B. bigemina*	4/4	1.0 (1-1)	0.1 (0.01-0.3)	39.9 (39.6-40.4)	24 (21-27)	1:380 (1:320-1:640)	947 (875-1036)	0
IV	60 kRad	1×10^{10} *B. bigemina*	4/4	1.5 (1-2)	0.03 (0.01-0.06)	39.9 (39.7-40.1)	25 (23-27)	1:269 (1:160-1:640)	938 (750-1143)	0
V	60 kRad (Frozen)	1×10^{10} *B. bigemina*	2/2	1.0 (1-1)	0.06 (0.05-0.07)	40.0 (40.0-40.0)	23 (21-25)	1:640 (1:640-1:640)	822 (768-875)	1[c]

[a] Characteristics of reactions based on data collected for 28 days post challenge.

[b] Range of means.

[c] Death due to acute anaphylaxis following challenge.

[d] Death due to acute babesiosis following challenge.

The average maximum parasitemias were 3.2 and 0.03% for calves infected with 1 x 10^{10} and 1 x 10^{7} nonirradiated B. bigemina parasitized erythrocytes, respectively. The average maximum parasitemias were 0.11 and 0.002% for calves infected with 1 x 10^{10} B. bigemina parasitized erythrocytes exposed to 24 and 36 kRad, respectively.

The average maximum morning rectal temperatures were 41.5 and 40.5°C for calves infected with 1 x 10^{10} and 1 x 10^{7} nonirradiated B. bigemina parasitized erythrocytes, respectively. The average maximum morning rectal temperatures were 40.3 and 40.1°C for calves infected with 1 x 10^{10} B. bigemina parasitized erythrocytes exposed to 24 and 36 kRad, respectively.

The average minimum packed cell volumes were 18 and 27% for calves infected with 1 x 10^{10} and 1 x 10^{7} nonirradiated B. bigemina parasitized erythrocytes, respectively. The average minimum packed cell volumes were 26 and 30% for calves infected with 1 x 10^{10} B. bigemina parasitized erythrocytes exposed to 24 and 36 kRad, respectively.

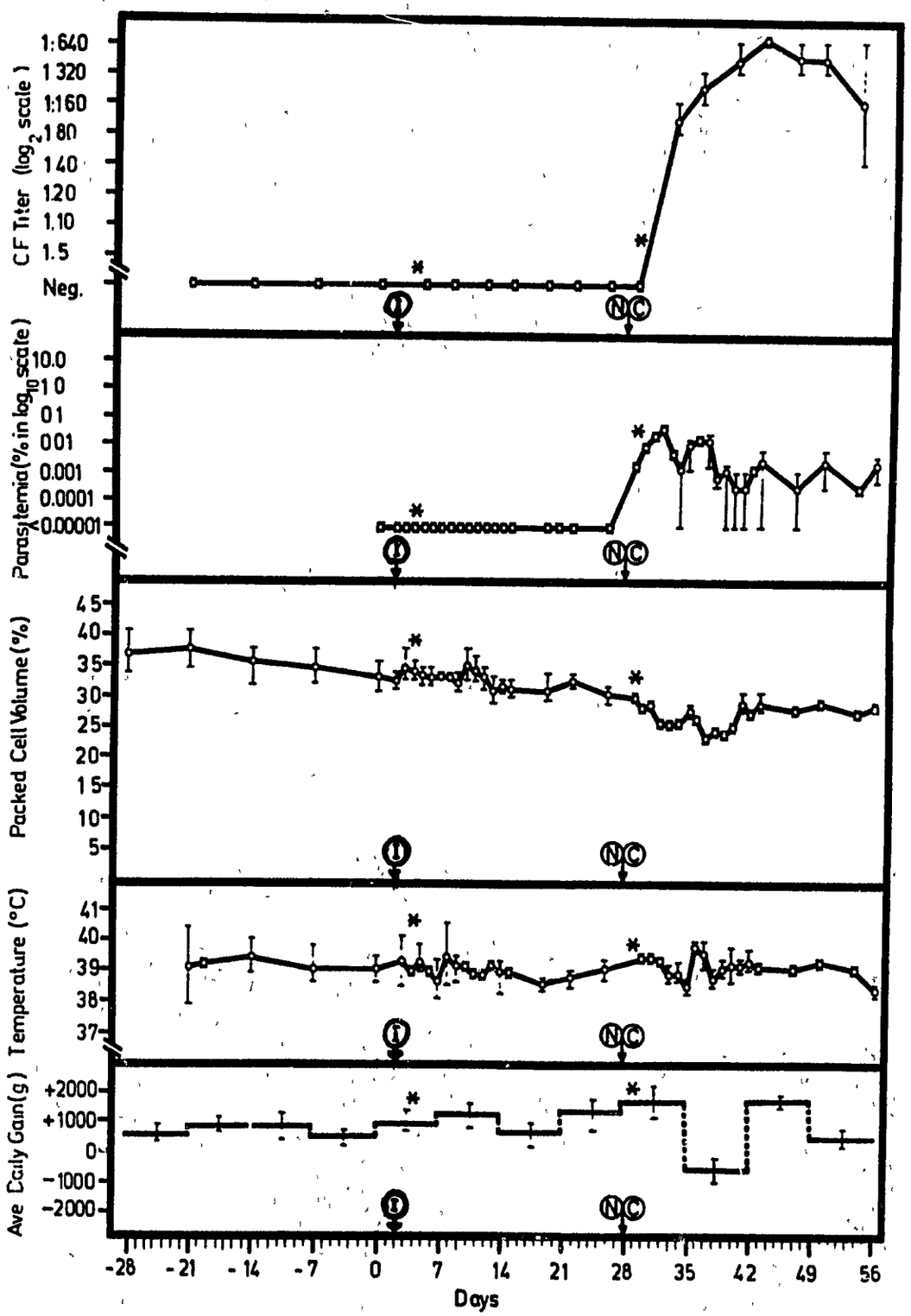
The average maximum CF titers were 1:320 and 1:201 for calves infected with 1 x 10^{10} and 1 x 10^{7} nonirradiated B. bigemina parasitized erythrocytes, respectively. The average maximum CF titers were 1:135 and 1:80 for calves infected with 1 x 10^{10} B. bigemina parasitized erythrocytes exposed to 24 and 36 kRad, respectively.

The average daily gains were 423 and 798 g for 28 days following inoculation with 1×10^{10} and 1×10^{7} nonirradiated B. bigemina parasitized erythrocytes, respectively. The average daily gains were 661 and 857 g for calves infected with 1×10^{10} B. bigemina parasitized erythrocytes exposed to 24 and 36 kRad, respectively.

The fifth experiment was undertaken to determine the effect of gamma radiation of 60 kRad and freezing on the infectivity and immunogenicity of B. bigemina in calves. The results of experiment V summarized in Figure 24 and Table 3 (p. 107) show that calves inoculated with B. bigemina parasitized blood exposed to 60 kRad and frozen did not develop progressive parasitemias.

The results of experiments IV and V show that all calves developed progressive parasitemias with similar prepatent periods following challenge with 1×10^{10} nonirradiated B. bigemina parasitized erythrocytes. The average maximum parasitemias were 0.006, 0.004, 0.5, 0.8, and 0.6% following challenge for calves inoculated 4 weeks previously with 1×10^{10} nonirradiated B. bigemina, 1×10^{7} nonirradiated B. bigemina, 1×10^{4} nonirradiated B. bigemina, and 10 ml normal blood irradiated at 36 kRad, respectively. One calf inoculated 4 weeks before challenge with 12 ml normal blood irradiated at 36 kRad died from acute babesiosis following challenge with 1×10^{10} nonirradiated B. bigemina parasitized erythrocytes. The average maximum parasitemias were 0.003, 0.02, 0.1, 0.03, and 0.06% following challenge for calves inoculated 4 weeks

Figure 24. Complement fixation titers, % parasitized erythrocytes, % packed cell volumes, morning rectal temperatures and average daily gains for 4 calves inoculated (I) with 1 x 10^{10} B. bigemina parasitized erythrocytes exposed to 60 kRad and frozen, and challenged (C) 4 weeks later with 1 x 10^{10} B. bigemina parasitized erythrocytes. The calves were negative (N) on subinoculation the day of challenge. Two calves died (*) with severe systemic reactions, one following inoculation and the other following challenge.


CF Titer (log2 scale)
1:640
1 320
1:160
1 80
1 40
1 20
1.10
1.5
Neg.
Parasitemia (% in log10 scale)
10.0
1 0
0 1
0 01
0.001
0.0001
<0.00001
Packed Cell Volume (%)
45
40
35
30
25
20
15
10
5
Temperature (°C)
41
40
39
38
37
Ave Daily Gain (g)
+2000
+1000
0
−1000
−2000
I
N
C
-28
-21
-14
-7
0
7
14
21
28
35
42
49
56
Days

previously with 1 x 10^{10} B. bigemina irradiated at 24, 36, 48, 60, and 60 kRad (frozen), respectively.

The average maximum morning rectal temperatures were 40.2, 40.2, 41.3, 41.1, and 41.4°C following challenge for calves inoculated 4 weeks previously with 1 x 10^{10} nonirradiated B. bigemina, 1 x 10^{7} nonirradiated B. bigemina, 1 x 10^{4} nonirradiated B. bigemina, 1 x 10^{10} heat inactivated B. bigemina, and 10 ml normal blood irradiated at 36 kRad, respectively. The average maximum morning rectal temperatures were 39.7, 40.0, 39.9, 39.9, and 40.0°C following challenge for calves inoculated 4 weeks previously with 1 x 10^{10} B. bigemina irradiated at 24, 36, 48, 60, and 60 kRad (frozen), respectively.

The average minimum packed cell volumes were 29, 28, 11, 19, and 10% following challenge for calves inoculated 4 weeks previously with 1 x 10^{10} nonirradiated B. bigemina, 1 x 10^{7} nonirradiated B. bigemina, 1 x 10^{4} nonirradiated B. bigemina, 1 x 10^{10} heat inactivated B. bigemina and 10 ml normal blood irradiated at 36 kRad, respectively. The average minimum packed cell volumes were 27, 26, 24, 25, and 23% following challenge for calves inoculated 4 weeks previously with 1 x 10^{10} B. bigemina irradiated at 24, 36, 48, 60, and 60 kRad (frozen), respectively.

The average maximum CF titers were 1:201, 1:127, 1:160, 1:640, and 1:227 following challenge for calves inoculated 4 weeks previously with 1 x 10^{10} nonirradiated B. bigemina, 1 x 10^{7} nonirradiated B. bigemina, 1 x 10^{4} nonirradiated B. bigemina, 1 x 10^{10}

heat inactivated B. bigemina and 10 ml normal blood irradiated at 36 kRad, respectively. The average maximum CF titers were 1:134, 1:201, 1:380, 1:269, and 1:640 following challenge for calves inoculated 4 weeks previously with 1 x 10^{10} B. bigemina irradiated at 24, 36, 48, 60, and 60 kRad (frozen), respectively.

The average daily gains were 887, 976, 813, 815, and 982 g for 28 days following challenge for calves inoculated 4 weeks previously with 1 x 10^{10} nonirradiated B. bigemina, 1 x 10^{7} nonirradiated B. bigemina, 1 x 10^{4} nonirradiated B. bigemina, 1 x 10^{10} heat inactivated B. bigemina, and 10 ml normal blood irradiated at 36 kRad, respectively. The average daily gains were 1093, 1060, 947, 938, and 822 g for 28 days following challenge for calves inoculated 4 weeks previously with 1 x 10^{10} B. bigemina irradiated at 24, 36, 48, 60, and 60 kRad (frozen), respectively.

One calf in experiment V developed a severe systemic reaction following inoculation with previously frozen B. bigemina parasitized blood exposed to 60 kRad. Within 2 minutes of completion of the injection, the calf suffered intense respiratory distress, collapsed to its side, and showed nystagmus. The calf died 36 hours following injection. Post-mortem examination of the calf revealed lesions which were confined largely to the repiratory system. The lungs had severe intra-alveolar and interstitial edema and emphysema with intra-alveolar hemorrhage. The trachea and major bronchi contained a cream colored frothy fluid. Submucosal petechia were present in the trachea. Visceral and

parietal subpleural petechia were numerous. The nasal turbinate mucosa was congested as were the bronchial and mediastinal lymph nodes.

Six calves in experiments IV and V developed severe systemic reactions within 2 minutes following challenge with blood containing 1×10^{10} nonirradiated B. bigemina parasitized erythrocytes. Three of these calves (one inoculated 4 weeks before challenge with 1×10^{10} B. bigemina irradiated at 22 kRad, one inoculated 4 weeks before challenge with 1×10^{10} B. bigemina irradiated at 60 kRad, and one inoculated with 10 ml normal blood irradiated at 36 kRad) suffered intense respiratory distress. These calves breathed more easily after 15 minutes. Their postures were characteristic of dyspneic animals with the neck extended, head lowered, mouth opened, and tongue protruded. Abdominal respiratory movements were marked and grunting accompanied expirations. The other 3 calves (one inoculated 4 weeks before challenge with 1×10^{10} B. bigemina irradiated at 36 kRad, one inoculated 4 weeks before challenge with 1×10^{4} nonirradiated B. bigemina and one inoculated 4 weeks before challenge with 1×10^{10} B. bigemina previously irradiated at 60 kRad and frozen) suffered intense respiratory distress, collapsed, and showed nystagmus. Coughing produced cream colored frothy fluids mixed with blood. These calves died within 24 hours following injection. Post-mortem examination revealed lesions which were confined largely to the respiratory system, which included severe

intra-alveolar and interstitial edema and emphysema with intra-alveolar hemorrhage of the lung. The trachea and major bronchi contained a hemorrhagic cream colored frothy fluid.

DISCUSSION

The results of the experiment designed to obtain B. bigemina free from contaminating organisms show that B. argentina and B. bigemina were found in blood smears from the splenectomized calf 16 and 18 days, respectively, after the calf had been placed on a tick-infested pasture. Callow and Hoyte (1961b) observed the experimental transmission of B. bigemina by B. microplus and recorded patent infections with B. bigemina 11 to 17 days following the placement of larva on cattle and that transmission did not take place until ticks were nymphs. Callow and Hoyte (1961b) also reported that B. argentina could be transmitted by larva and therefore patent infections with B. argentina could be detected earlier than patent infections with B. bigemina following placement of larva on cattle. The observations on the natural transmission of B. bigemina in the present experiment are in agreement with the observations of the experimental transmission of B. bigemina by B. microplus recorded by Callow and Hoyte (1961b).

The ability of B. bigemina to multiply so rapidly that it was found in thin blood smears from 24 to 57½ hours after calves had been inoculated with relatively small numbers of parasites, made it possible to separate B. bigemina from other blood parasites. The prepatent periods for B. bigemina observed were in agreement with those prepatent periods reported by Callow and Hoyte (1961a).

The results obtained in studies designed to determine the minimum irradiation dosage which would inhibit multiplication of B. bigemina indicate that calves infected with B. bigemina parasitized erythrocytes exposed to doses up to and including 30 kRad developed progressive parasitemias. Some calves receiving parasitized erythrocytes irradiated at levels of 36 and 42 kRad did not develop progressive infections. Progressive infections were prevented by exposure to irradiation at 48 kRad or higher. Subinoculations into susceptible splenectomized calves from parasites thus treated failed to produce active infections. These results confirm and extend those reported recently from cattle inoculated with irradiated B. bigemina (Castro & Canabez, 1968) and from mice and rats inoculated with irradiated B. rodhaini (Phillips, 1970 & 1971b).

The results also showed that in addition to a lower infection rate brought about by irradiated parasites, calves that did become infected had prolonged prepatent periods and lower maximum parasitemias than control calves receiving nonirradiated inocula. The results could be due to the death or developmental arrest of some of the parasites, or to an overall retarded development leading to more slowly developing forms. Control calves that had been inoculated with 1×10^7 nonirradiated parasitized erythrocytes in experiment IV also had prolonged prepatent periods and lower maximum parasitemias when compared to control calves inoculated with 1×10^9 and 1×10^{10} nonirradiated parasitized erythrocytes.

Therefore, the prolonged prepatent periods and lower maximum parasitemias in calves that had received irradiated parasites could have been due in part to a reduction in the number of viable parasites injected.

The results of experiment IV in which control calves were inoculated with 1 x 10^7 parasitized erythrocytes indicate that a reduction in parasitemia may have been the result of the reduction in the number of viable parasites injected and that such a standardized inoculum might be of use as a vaccine to produce an attenuated infection. Callow and Mellors (1966) have developed a similar monovalent vaccine for B. argentina prepared in splenectomized calves. Kemron et al. (1964) and Pipano (1969) have also reported that the length of incubation period was inversely related and the severity of the reaction directly related to the number of parasites in the inoculum.

An analysis of the foregoing experiments indicates that a degree of acquired resistance of infection with B. bigemina developed in calves after 1 inoculation with B. bigemina parasitized blood irradiated at 48 and 60 kRad. The resistance developed was sufficient to suppress multiplication of the Babesia and to permit calves to survive otherwise severe clinical infections with nonirradiated parasites. There was also less erythrocytic destruction (indicated by a decrease in the packed cell volumes) and a smaller increase in rectal temperatures following challenge for calves previously inoculated with B. bigemina parasitized

blood irradiated at 48 and 60 kRad. Presumably the irradiated parasites were responsible for the development of this resistance since irradiated nonparasitized blood did not produce a discernable acquired resistance to B. bigemina. These results are in agreement with and extend those recently reported from mice and rats immunized with irradiated B. rodhaini (Phillips, 1970 & 1971b).

The failure of the immunized calves to develop patent infections following inoculation of irradiated parasites, and the fact that blood taken on the day of challenge from these calves failed to produce infections when subinoculated into splenectomized calves indicate that immunity could not be considered to have been developed in response to a long standing chronic blood infection. The observation that inoculation of calves with irradiated nonreplicating B. bigemina 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 (Callow, 1968), B. bigemina (Callow, 1964 & 1967), B. divergens (Joyner & Davies, 1967), B. microti (Cox & Young, 1969), and B. rodhaini (Cox & Young, 1969; Phillips, 1969 & 1971b).

A similar degree of acquired resistance to infection with B. bigemina developed in calves inoculated with 1×10^{10} B. bigemina irradiated at 48 and 60 kRad and in calves inoculated with 1×10^{10}

nonirradiated B. bigemina. It seems likely that the protective immunity produced with irradiated nonreplicating B. bigemina may be similar to that produced with living pathogenic B. bigemina in non-fatal infections.

The acquired resistance to infection with B. bigemina developed in calves inoculated with 1×10^{10} B. bigemina irradiated at 48 and 60 kRad was much greater than the acquired resistance to infection developed in calves inoculated with 1×10^{10} heat killed B. bigemina. Thus, it seems likely that immunization with irradiated nonreplicating Babesia may provide the special immunological properties of living parasites important for producing a strong immunity while suppressing the pathogenic effects of the parasite.

Although CF antibodies are probably not functional in immunity (Mahoney, 1967a), it was of interest for theoretical and diagnostic considerations to determine whether detectable CF antibodies were produced in calves after one inoculation with B. bigemina parasitized blood irradiated at 48 and 60 kRad. The results of the CF microtiter tests indicated that 3 out of 8 calves were able to produce detectable levels of antibodies with maximum titers ranging from 1:5 to 1:20 even when the normal development and multiplication of Babesia were interrupted by irradiation. There was no discernable difference in acquired resistance to infection between those calves which did or did not develop detectable CF antibodies.

The success obtained in immunizing calves with B. bigemina which had been irradiated, frozen, and stored in a dry ice cabinet may be of practical significance in future studies. The Babesia parasites could be irradiated and frozen, without apparent loss of immunizing properties, for use in distant parts of the world.

It is of interest to note a consistant temperature rise in calves following intravenous inoculations with B. bigemina even when the normal development and multiplication of Babesia were interrupted by irradiation or heat inactivation. Vaccines which contain foreign protein can act as pyrogens that cause body temperature rise following injection (Guyton, 1961). It is also possible that plasma proteins and other proteins in blood which are not normally pyrogenic or toxic could be changed chemically by hemolysis, degradation, or denaturization and thereafter cause systemic reactions (Guyton, 1961). One calf died following the development of a severe systemic toxic reaction after intravenous inoculation with previously frozen B. bigemina parasitized blood exposed to 60 kRad.

Nine of 51 calves also developed severe systemic reactions following intravenous challenge with blood containing 1×10^{10} nonirradiated B. bigemina. These calves had previously received intravenous injections of irradiated or nonirradiated B. bigemina parasitized blood. Aitken and Sanford (1969) have recently described similar anaphylactic reactions in calves given 1 intravenous

injection of a foreign protein following challenge by the same route after a latent period of at least 7 days. Anaphylactic shock of this type however, usually only follows the intravenous injection of the challenge dose of antigen and could probably be avoided by using other routes in which the antigen would reach the circulation more slowly.

Standardized infecting and immunizing doses in the present study were given intravenously because previous studies have indicated that Babesia may be more immunogenic when given by the intravenous route(Mahoney,1967b). The same standardized inocula were given intravenously for challenge since the infections produced by these inocula given by the intravenous route had already been characterized. In view of the above observations,a recharacterization of challenge inoculum using a subcutaneous route of inoculation would be warranted for future studies using infected blood for challenge. Experimental or natural field tick challenge would also most likely avoid the problem of anaphylactic reactions following challenge in calves previously inoculated with irradiated B. bigemina parasitized blood.

SUMMARY

Experiments were carried out to separate B. bigemina from B. argentina, B. major, and A. marginale. The method of separation was rapid passage through 5 splenectomized calves. Five blood passages were carried out in 6 ½ days. Babesia argentina, B. major, and A. marginale were eliminated as contaminants after 4 passages. A frozen stabilate of the isolated B. bigemina was established.

A method for the preparation and examination of combination thin and thick blood films for the detection of Babesia parasitemia was developed. The technique for the staining of the combination thin and thick films involved the use of a phosphate buffered Giemsa stain solution containing alkyl phenoxy polyethoxy ethanol. Babesial CF antigens were also prepared and titrated for use in a CF microtiter procedure.

Babesia bigemina parasitized blood exposed to varied doses of gamma radiation up to 60 kRad was inoculated into calves. Calves infected with 1×10^{10} B. bigemina parasitized erythrocytes exposed to doses up to and including 30 kRad developed progressive parasitemias. Some calves receiving 1×10^{10} parasitized erythrocytes irradiated at levels of 36 and 42 kRad did not develop progressive infections. Progressive infections were prevented by exposure to irradiation at 48 kRad or higher. Subinoculation into susceptible splenectomized calves from parasites thus treated failed to produce active infections.

In addition to a lower infection rate brought about by irradiated parasites, calves that did become infected had prolonged prepatent periods and lower maximum parasitemias. Control calves, however, that had been inoculated with 1×10^7 nonirradiated parasitized erythrocytes also had prolonged prepatent periods and lower maximum parasitemias. Therefore, the prolonged prepatent periods and lower maximum parasitemias in calves that had received 1×10^{10} irradiated B. bigemina could have been due in part to a reduction in the number of viable parasites injected. The finding that calves inoculated with 1×10^7 nonirradiated parasitized erythrocytes had prolonged prepatent periods and lower maximum parasitemias indicate that such a standardized inoculum might be of use as a vaccine to produce an attenuated infection.

A degree of acquired resistance to infection with B. bigemina developed in calves after 1 inoculation with B. bigemina parasitized blood irradiated at 48 and 60 kRad. The resistance was sufficient to suppress multiplication of the Babesia and to permit calves to survive otherwise severe clinical infections with nonirradiated parasites. There was also less erythrocytic destruction and a smaller increase in rectal temperatures following challenge. Presumably the irradiated parasites were responsible for the development of resistance since irradiated nonparasitized blood did not produce a discernable acquired resistance. The observation that inoculation of calves with irradiated B. bigemina stimulated a degree of protective immunity, suggests that the

presence of replicating Babesia in the host is not necessary for the development of acquired resistance.

A similar degree of acquired resistance to infection with B. bigemina developed in calves inoculated with 1 x 10^{10} B. bigemina irradiated at 48 and 60 kRad and in calves inoculated with 1 x 10^{10} nonirradiated B. bigemina. It seems likely that the protective immunity produced with irradiated B. bigemina may be similar to that produced with living pathogenic B. bigemina in nonfatal infections. The acquired resistance to infection with B. bigemina developed in calves inoculated with 1 x 10^{10} B. bigemina irradiated at 48 and 60 kRad was much greater than the acquired resistance to infection developed in calves inoculated with 1 x 10^{10} heat killed B. bigemina. Thus, it seems likely that immunization with irradiated nonreplicating Babesia may provide the special immunological properties of living parasites important for producing a strong immunity while suppressing the pathogenic effects of the parasite.

The success obtained in immunizing calves with B. bigemina which had been irradiated, frozen, and stored in a dry ice cabinet may be of practical significance in future studies. The Babesia parasites could be irradiated and frozen, without loss of immunizing properties, for use in distant parts of the world.

BIBLIOGRAPHY

Aitken, M. M. & Sanford, J. (1969) Experimental Anaphylaxis in Cattle. J. Comp. Path. 79, 131

Anon. (1939) Ann. Rept. Qd. Dept. Agric. and Stock, Qd. Govt. Printer, Brisbane, Australia

Anon. (1956) Ann. Rept. Qd. Dept. Agric. and Stock, Qd. Govt. Printer, Brisbane, Australia

Anon. (1958) A Manual for Conducting the Complement-Fixation Test for Anaplasmosis. Agricultural Research Service, U.S. Department of Agriculture, Washington, D.C.

Anon. (1966) Nutrient Requirements of Dairy Cattle, 3rd Ed., National Research Council, National Academy of Sciences, Pub. 1349, Washington, D.C.

Anon. (1968) Isotopes and Radiation in Parasitology, International Atomic Energy Agency, Tech. Rpt. Series, 181, Vienna

Anon. (1970) Manual for the Laboratory Diagnosis of Parasitic Diseases II, Blood Parasites 8010-C. Parasitology Training Unit, National Communicable Disease Center, Atlanta, Georgia

Barnett, S. F. (1964) The Preservation of Babesia bigemina, Anaplasma centrale, and A. marginale by Deep Freezing. Vet. Rec. 76, 4

Bennison, B. E. & Coatney, G. R. (1945) Inactivation of Malarial Parasites by X-rays. Public Health Reports 60, 127

Bridgman, J. & Kimball, R. F. (1954) The Effects of X-rays on Division Rate and Survival of Tillina magna and Colpoda sp. with an Account of Delayed Death. J. Cell. and Comp. Physiol. 44, 431

Briggs, N. T., Garza, B. L. & Box, E. D. (1960) Alterations of Serum Proteins in Mice Acutely and Chronically Infected with Plasmodium berghei. Exp. Parasit. 10, 21

Briggs, N. T. & Wellde, B. T. (1969) Some Characteristics of Plasmodium berghei "Relapsing" in Immunized Mice. Mil. Med. 134, 1243

Callow, L. L. (1964) Strain Immunity in Babesiosis. Nature 204, 1213

Callow, L. L. (1967) Sterile Immunity, Coinfectious Immunity, and Strain Differences in Babesia bigemina Infections. Parasit. 57, 455

Callow, L. L. (1968) A Note on Homologous Strain Immunity in Babesia argentina Infections. Aust. Vet. J. 44, 268

Callow, L. L. & Hoyte, H. M. (1961a) The Separation of Babesia bigemina from Babesia argentina and Theileria mutans. Aust. Vet. J. 37, 66

Callow, L. L. & Hoyte, H. M. (1961b) Transmission Experiments using Babesia bigemina, Theileria mutans, Borrelia sp. and the Cattle Tick, Boophilus microplus. Aust. Vet. J. 37, 381

Callow, L. L. & Mellors, L. T. (1966) A New Vaccine for Babesia argentina Infection Prepared in Splenectomized Calves. Aust. Vet. J. 42, 464

Callow, L. L. & Tammemagi, L. (1967) Vaccination against Bovine Babesiosis: Infectivity and Virulence of Blood from Animals either Recovered from or Reacting to Babesia argentina. Aust. Vet. J. 43, 249

Castro, E. R. & Canabez, F. (1968) Propiedades Biologicas y Caracteristicas de Babesia bigemina. Efectos de Radiaciones Ionicas sobre la Infeccionidad de Sangre Total Infectada. Bol. Chileno Parasit. 23, 30

Ceithaml, J. & Evans, E. A., Jr. (1946) The Biochemistry of the Malaria Parasite. IV The in vitro Effects of X-rays upon Plasmodium gallinaceum. J. Infect. Dis. 78, 190

Corradetti, A., Verolini, F., & Bucci, A. (1966) Resistanza a Plasmodium berghei de parte de Ratti Albini Precedentement Immunizzati con Plasmodium berghei Irradiato. Parassitologia 8, 133

Corradetti, A., Verolini, F., & Bucci, A. (1968) Comportamento de Topini Bianchi Immunizzati con Plasmodium berghei Irradiato, di Fronte Alla Reinoculazione con Ceppo Omologo e Alla Reinoculazione con Ceppo Eterologo. Parassitologia 10, 113

Cox, F. E. G. & Young, A. S. (1969) Acquired Immunity to Babesia microti and Babesia rodhaini in mice. Parasit. 59, 257

Crowther, J. A. (1926) The Action of X-rays on Colpidium colpoda. Proc. Roy. Soc., London 100, 390

Dalgleish, R. J. (1968) Field Observations on Babesia argentina Vaccination in Queensland. Aust. Vet. J. 44, 103

Duxbury, R. E. & Sadun, E. H. (1969) Resistance Produced in Mice and Rats by Inoculation with Irradiated Trypanosoma rhodeniense. J. Parasit. 55, 859

Duxbury, R. E. & Sadun, E. H. (1970) Isotopes and Radiation in Parasitology II, p 83. International Atomic Energy Agency, Tech. Rpt. Series, 242, Vienna

Frerichs, W. M., Johnson, A. J., & Holbrook, A. A. (1968) Storage of Babesia caballi and Babesia equi in Liquid Nitrogen. J. Parasit. 54, 451

Frerichs, W. M., Holbrook, A. A. & Johnson, A. J. (1969) Equine Piroplasmosis: Production of Antigens for the Complement Fixation Test. Am. J. Vet. Res. 30, 1337

Giese, A. C. (1967) Research in Protozoology, Ed. T. T. Chen, p. 267. Pergamon Press, Oxford and London

Guyton, A. C. (1961) Medical Physiology, p. 964. W. B. Saunders Company, Philadelphia and London

Hance, R. T. & Clark, H. (1926) Studies on X-ray Effects: XIV. The Effects of X-rays on the Division Rate of Paramecium. J. Exp. Med. 43, 61

Hidalgo, R. J. & Dimopoullos, G. T. (1967) Complement-Fixation Microprocedures for Anaplasmosis. Am. J. Vet. Res. 28, 245

Hirato, K., Ninomiya, N., Uwano, Y. & Kutu, T. (1945) Studies on The Complement Fixation Reaction for Equine Piroplasmosis. Jap. J. Vet. Sci. 7, 197

Irvin, A. D. & Brocklesby, D. W. (1969) Continuous High Parasitemia in Mice Infected with Babesia (Nuttallia) microti. J. Parasit. 55, 1190

Johnson, A. J., Frerichs, W. M. & Holbrook, A. A. (1969) Equine Piroplasmosis: Complement Fixation Titers of Horses Infected with Babesia caballi. Am. J. Vet. Res. 30, 697

Joyner, L. P. & Davies, S. F. M. (1967) Acquired Resistance to Babesia divergens in Experimental Calves. J. Protozool. 14, 260

Kemron, A., Hadani, A., Egyed, M., Pipano, E., & Neuman, M. (1964) Studies on Bovine Piroplasmosis Caused by Babesia bigemina: III. The Relationship between the Number of Parasites in the Inoculum and the Severity of the Response. Ref. Vet. 21, 108

Kimball, R. F. (1955) Radiation Biology, Ed. A. Hollaender, p. 285. McGraw-Hill Co., New York

Legg, J. (1928) Some Notes on Babesiosis (Babesia bigemina) of Cattle. Aust. Vet. J. 4, 98

Legg, J. (1931) The Value of the Blood of Recovered Cattle in Redwater Inoculation. Aust. Vet. J., 7, 70

Legg, J. (1939) Recent Observations on the Premunization of Cattle against Tick-fevers in Queensland. Aust. Vet. J. 15, 46

Lumsden, W. H. & Hardy, G. J. (1965) Nomenclature of Living Parasite Material. Nature 205, 1032

Mahoney, D. F. (1962a) The Epidemiology of Babesiosis in Cattle. Aust. J. Sci. 24, 210

Mahoney, D. F. (1962b) Bovine Babesiosis: Diagnosis of Infection by a Complement-Fixation Test. Aust. Vet. J. 38, 48

Mahoney, D. F. (1964) Bovine Babesiosis: An Assessment of the Significance of Complement Fixing Antibody based upon Experimental Infection. Aust. Vet. J. 40, 369

Mahoney, D. F. (1967a) Bovine Babesiosis: The Passive Immunization of Calves against Babesia argentina with Special Reference to the Role of Complement Fixing Antibodies. Exp. Parasit. 20, 119

Mahoney, D. F. (1967b) Bovine Babesiosis: The Immunization of Cattle with Killed Babesia argentina. Exp. Parasit. 20, 125

Mahoney, D. F. (1967c) Bovine Babesiosis: Preparation and Assessment of Complement Fixing Antigens. Exp. Parasit. 20, 232

Mahoney, D. F. & Saal, J. R. (1961) Bovine Babesiosis: Thick Blood Films for the Detection of Parasitemia. Aust. Vet. J. 37, 44

Nussenzweig, R. W., Vanderberg, J., Most, H., & Orton, C. (1967) Protective Immunity Produced by the Injection of X-irradiated Sporozoites of Plasmodium berghei. Nature 216, 160

Nussenzweig, R., Vanderberg, J., & Most, H. (1969a) Protective Immunity Produced by the Injection of X-irradiated Sporozoites of Plasmodium berghei: IV. Dose Response, Specificity, and Humoral Immunity. Mil. Med. 134, 1176

Nussenzweig, R. S., Vanderberg, J. P. Most, H., & Orton, C. (1969b) Specificity of Protective Immunity Produced by X-irradiated Plasmodium berghei Sporozoites. Nature 222, 488

Ord, M J. & Danielli, J. F. (1956) The Site of Damage in Amoebae Exposed to X-rays. Quart. J. Micro. Sci. 97, 29

Overdulve, J. P. & Antonisse, H. W. (1970) Measurement of the Effect of Low Temperature on Protozoa by Titration. II. Titration of Babesia rodhaini, Using Prepatent Period and Survival Time, Before and After Storage at -76°C. Exp. Parasit. 27, 323

Palmer, J. G., Eichwald, E. J., Cartwright, G. E., & Wintrobe, M. M. (1953) The Experimental Production of Splenomegaly, Anemia and Leukopenia in Albino Rats. Blood 8, 72

Phillips, R. S. (1969) Babesia rodhaini Infections in the Rat after Acute Primary Parasitemia. Parasit. 59, 349

Phillips, R. S. (1970) Resistance of Mice and Rats to Challenge by Babesia rodhaini after Inoculation with Irradiated Red Cells Infected with B. rodhaini. Nature 227, 1255

Phillips, R. S. (1971a) Evidence that Piroplasms can Undergo Antigenic Variation. Nature 231, 323

Phillips, R. S. (1971b) Immunity of Rats and Mice Following Injection with Co-60 Irradiated Babesia rodhaini Infected Red Cells. Parasit. 62, 221

Pipano, E. (1969) Immunization of Cattle against Babesiella berbera Infection: I. Infection of Cattle with Blood from Patent and Latent Carriers. Ref. Vet. 26, 11

Pipano, E. & Senft, Z. (1966) Storage of Babesia bigemina, Babesiella berbera, Anaplasma marginale, and Anaplasma centrale in the Deep Freeze. J. Protozool. 13, (Supplement) 34

Reusse, U. (1956) Konservierung Einiger Tierpathogener Protozoen durch Aufbewahrung bei tiefen Temperaturen. Tropenmed. Parasit. 7, 99

Rigdon, R. H. & Rudisell, H., Jr. (1945) Effect of Radiation on Malaria. An Experimental Study in the Chick and Duck. Proc. Soc. Exp. Biol. Med. 59, 167

Sadun, E. H., Wellde, B. T. & Hickman, R. L. (1969) Resistance Produced in Owl Monkeys (Aotus trivirgatus) by Inoculation with Irradiated Plasmodium falciparum. Mil. Med. 134, 1165

Sanders, A. & Wallace, F. G. (1966) Immunization of Rats with Irradiated Trypanosoma lewisi. Exp. Parasit. 18, 301

Schindler, R. & Denning, H. K. (1962) Demonstration of Babesia Antibodies by the Complement Fixation Reaction. Z. Tropenmed. Parasit. 13, 480

Sergent, E., Donatien, A., Parrot, L., & Lestoquard, F. (1945) Etudes sur les Piroplasmoses Bovines. Institut Pasteur d'Algerie, Alger

Sergent, E., Donatien, A., Parrot, L., Lestoquard, F., & Plantureux, E. (1927) Etudes Experimentales sur les Piroplasmoses Bovines d'Algerie. Ann. Inst. Pasteur 41, 721

Sergent, E., Parrot, L., & Donatien, A. (1924) Une Question de Terminologie: Immuniser et Premunir. Bull. Soc. Path. Exot. 17, 37

Setlow, R. B. & Polland, R. C. (1962) Molecular Biophysics. Addison-Wesley Publ. Co., Reading, Mass.

Sinton, J. A. (1939) Studies in Immunity in Malaria: IV. The Effect of Drugs upon the Development of Immunity and its Relationship to the Principles of Treatment. J. Malaria Inst. India 2, 191

Seddon, H. R. (1966) Diseases of Domestic Animals in Australia. IV. Protozoan and Virus Diseases. p. 25. Commonwealth of Australia Department of Health, Commonwealth Government Printer, Canberra

Summerville, W. A. T. (1960) Queensland Annual Report of the Department of Agriculture and Stock. Government Printer, Brisbane, Australia

Targett, G. A. T. & Fulton, J. D. (1965) Immunization of Rhesus Monkeys against Plasmodium knowlesi malaria. Exp. Parasit. 17, 180

Targett, G. A. T. (1968) Immunity to Parasites, Ed. A. E. R. Taylor, p. 27. Blackwell Scientific Publication, Oxford and Edinburgh

Tidswell, F. (1899) Report on Protective Inoculation against Tick Fever. Dept. Pub. Health, N.S.W., Govt. Printer, Sydney, Australia

Tidswell, F. (1900) Second Report on Protective Inoculation against Tick Fever. Dept. Pub. Health, N.S.W., Govt. Printer, Sydney, Australia

Todorovic, R. A. & Adams, L. G. (1971) Serodiagnosis of Babesiosis. Sci. Proc. XIX World Veterinary Congress, Mexico City, p. 1114

Tsur, I. (1961) Immunization Trials against Bovine Babesiosis: I. Vaccination with Blood from Latent Carriers. Ref. Vet. 18, 103

Tsur, I. (1962) Immunization Trials against Bovine Babesiosis: II. Vaccination with Blood from 'Patent" Carriers. Ref. Vet. 19, 181

Vanderberg, J. P., Nussenzweig, R. S., Most, H., & Orton, C. G. (1968) Protective Immunity Produced by the Injection of X-irradiated Sporozoites of Plasmodium berghei. II. Effects of Radiation on Sporozoites. J. Parasit. 54, 1175

Waddell, A. H. (1963) Deep Freeze Storage of Babesia bigemina. Aust. Vet. J. 39, 400

Ward, R. A., Bell, L. H. & Schneider, R. L (1960) Effects of X-irradiation on the Development of Malaria Parasites in Mosquitoes. Exp. Parasit. 10, 324

Weiss, M. L. (1968) Active Immunization of Mice against Plasmodium berghei: Cross Resistance to a Recently Isolated Strain. Am. J. Trop. Med. 17, 516

Weiss, M. L. & DeGiusti, D. L. (1965) The Effect of Different Sera in the Culture Medium on the Behavior of Plasmodium berghei following Serial Passage through Tissue Culture. J. Protozool. 11, 224

Weiss, M. L. & DeGuisti, D. L. (1966) Active Immunization against Plasmodium berghei Malaria in Mice. Am. J. Trop. Med. 15, 472

Wellde, B. T. & Sadun, E. H. (1967) Resistance Produced in Rats and Mice by Exposure to Irradiated Plasmodium berghei. Exp. Parasit. 21, 310

Wellde, B. T., Ward, R. A., & Ueoka, R. (1969) Aspects of Immunity in Mice Inoculated with Irradiated Plasmodium berghei. Mil. Med. 134, 1153

Zuckerman, A., Hamburger, J. & Spira, D. (1967) Active Immunization of Rats with a Cell-Free Extract of the Erythrocytic Parasites of Plasmodium berghei. Exp. Parasit. 21, 84

APPENDIX

Appendix Table A1. Composition of the Concentrates and Minerals Fed Experimental Animals.

Shelled Corn	430 kg
Cottonseed Meal	300 kg
Wheat Bran	240 kg
Iodized Salt	10 kg
Bone Meal	10 kg
Trace Mineral Mix*	10 kg
	1000 kg

*Trace Mineral Mix:

Copper Sulfate	1.95%
Iron Sulfate	7.47%
Zinc Oxide	1.24%
Manganese Sulfate	3.09%
Cobalt Sulfate	0.20%
Potassium Iodide	0.07%
Wheat Bran	85.98%
	100.00%

VITA

John Philip Bishop was born on June 28, 1943 in Delaware, Ohio. He is one of eight children born to Mr. and Mrs. Robert J. W. Bishop. He graduated from Fairbanks Local High School in 1961.

In June 1961 he entered Ohio State University and was awarded degrees of Bachelor of Science in Agriculture in 1964, Doctor of Veterinary Medicine in 1967, and Master of Science in 1969 with majors in animal nutrition and reproductive physiology.

From 1967 to 1969 he completed an internship in international animal health and production through Purdue University at the Universidad Nacional del Sur, Bahia Blanca, Argentina sponsored by the Ford Foundation. In 1969 he was a Travel Scholar at Purdue University through the C.I.C. Graduate Exchange Program between Purdue University and Ohio State University.

In September 1969 he entered the Graduate School of Texas A&M University on a postdoctoral fellowship in Veterinary Microbiology and Tropical Veterinary Medicine supported by the Rockefeller Foundation and the United States Agency for International Development. He is a candidate for the degree of Doctor of Philosophy in Veterinary Microbiology.

He is married to the former Kathryn Kay Parrott and they have one son, Mark Robert. Dr. Bishop's permanent mailing address is: R. R. 3., Marysville, Ohio 43040.

The typist for this dissertation was Mrs. Kay P. Bishop.