THE IMMUNOGLOBULIN RESPONSE IN INTACT AND SPLENECTOMIZED CALVES INFECTED WITH *ANAPLASMA MARGINALE*

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Anaplasmosis is an infectious disease of cattle and other ungulates. The causal organism, Anaplasma marginale, is transmitted by blood-sucking arthropods. After a variable incubation period, the organisms appear in the blood as 'marginal bodies' in the erythrocytes. The intensity of parasitemia then increases rapidly, and a concurrent anemia develops, which may be fatal. Convalescence is accompanied by solid lifelong immunity, which is dependent on the persistence of the organism within the body. (For a full review of anaplasmosis, see Anon., 1966) Infective immunity such as this is a poorly understood phenomenon. It also occurs in other infectious anemias, e.g. babesiosis.

It has been known for some time that the splenectomized calf develops a more severe, highly fatal disease when infected with Anaplasma marginale (Norman, 1966). The reasons for this phenomenon are unknown, but it has been reported in many hematozoan infections.

A number of studies have implicated the spleen in the production of antibodies early in the response to various antigens, especially particulate ones, such as erythrocyte stromata (Taliaferro and Taliaferro, 1950). Since these early antibodies are found principally in the high molecular-weight immunoglobulins (IgM), some authors have inferred that the spleen is an important source of these macroglobulins

(Fitch and Wissler, 1965; Berlin, 1965).

Marked macroglobulinemia has been described in malaria (Abele et al., 1964) and in trypanosomiasis (Masseyeff and Lamy, 1966). Recently Murphy, Osebold and Aalund (1966a) reported that early complement-fixing antibodies to Anaplasma marginale were predominantly in the Igm fraction of the immunoglobulins, and that this IgM response appeared to be quite pronounced.

The present study was planned to investigate the influence of splenectomy on the kinetics of the immunoglobulin response to Anaplasma marginale infection, and to characterize the response in intact calves on a quantitative basis. It was postulated that the IgM response in splenectomized calves would be delayed and/or depressed, and that this might be a significant factor in the pathogenesis of the severe disease patterns observed in these animals.

CHAPTER II

REVIEW OF SELECTED LITERATURE

The Role of the Spleen in Antibody Formation

Introduction.

Within recent years it has become apparent that antibody activity is associated with a physicochemically heterogeneous group of proteins, known as the immunoglobulins. At present, four types of immunoglobulins have been recognized in man. These are IgG, IgA, IgM and IgD (Fahey, 1965), according to the recommended nomenclature (Anon., 1964).

There is now considerable evidence that most antigens elicit a complex antibody response in animals and man. This consists of a rapid, often transient, production of IgM antibodies, followed by a more prolonged synthesis of IgG (low molecular-weight) antibodies. The biological significance of this heterogeneity is still unknown, but it has been described with such diverse materials as proteins, phage and other viruses, and bacterial antigens (Uhr and Finkelstein, 1963; Bauer, Mathies and Stavitsky, 1963).

The relative proportions of these two immunoglobulins produced in response to an antigen appears to depend on several factors, including the age of the individual (Ellis and Smith, 1966) and the chemical nature of the antigen (Bauer, Mathies and Stavitsky, 1963). Some antigens seem incapable of eliciting IgG antibodies, e.g. salmonella O-

antigen (Bauer, Mathies and Stavitsky, 1963). The form in which an antigen is presented to the cells of the reticuloendothelial system is believed to be important, since particulate antigen preparations elicit a more marked IgM response than soluble antigens (Torrigiani and Roitt, 1965).

Tissues Involved in Immunoglobulin Synthesis.

It is well established that antibody formation occurs principally in the lymphoreticular tissues of the spleen and lymph nodes. The major cell type involved is undoubtedly the plasma cell (Fagraeus, 1948), but there is an increasing body of evidence to suggest that the small lymphocyte may also be involved. The cellular origin of specific types of immunoglobulins remains a subject of considerable debate.

There is little doubt that spleen cells can synthesize both IgM and IgG immunoglobulins (Friedman and Young, 1966; Chiappino and Pernis, 1964). However, some investigators have suggested that the spleen may be a more important source of macroglobulins than other lymphoid tissues (Fitch and Wissler, 1965; Berlin, 1965). Since most of the information on spleen function has resulted from studies on the effects of splenectomy, this subject will be explored in greater detail.

Effects of Splenectomy on Immunoglobulin Levels.

In adult rats subtotal splenectomy caused a fall in beta and gamma globulins (Anderson and Biering, 1964), whereas when newborn rats were splenectomized or thymectomized, no qualitative or quantitative changes in serum proteins were detected at the age of 8-9 weeks. Only adults that survived the combined operation developed hypogamma-2-globulinemia (Azar, 1964). In guinea pigs splenectomy led to a fall in beta-

globulins only. One of the beta-globulins involved was apparently the beta-macroglobulin (IgM) (Anderson, Grunnel and Clausen, 1965). Dimopoullos, Schrader and Foote (1959) reported that calves developed hypogammaglobulinemia following splenectomy.

Effects of Splenectomy on Antibody Formation.

Splenectomy produces complex sequelae, the effects being dependent on several criteria. The fact that these criteria are ill-defined has no doubt been the partial cause of the confusion that surrounds the subject.

Some of the factors that seem to influence the response of the splenectomized animal to an antigen, are the age and species of the individual, the chemical nature of the antigen, the route and frequency of immunization, and the time of splenectomy relative to the time of immunization. Some of these factors will be considered in greater detail.

Splenectomy has been observed to impair antibody formation in many species, including the monkey (Saslaw and Carlisle, 1964), the rabbit (Motohashi, 1922; Taliaferro and Taliaferro, 1950; Draper and Sussdorf, 1957), the rat (Rowley, 1950a; Winebright and Fitch, 1962), the mouse (Adler, 1965), and the chicken (Rosenquist and Wolfe, 1962).

The antibody response of the splenectomized animal may differ from that of unoperated controls in one of a number of ways. It may be virtually absent (Rowley, 1950a; Fitch, 1963), delayed and depressed (Taliaferro and Taliaferro, 1950; Adler, 1965; Berglund, 1956; Rosenquist and Wolfe, 1962), or merely delayed (Rosenquist and Wolfe, 1962). Some of this variation may reflect true interspecies differences. For example, in the rat splenectomy virtually abolished the

antibody response to sheep red blood cells (SRBC) (Rowley, 1950a). On the other hand, in the rabbit and in the mouse the antibody response to SRBC was only delayed and depressed in splenectomized animals (Taliaferro and Taliaferro, 1950; Adler, 1965). However, one cannot exclude the possibility that these differences resulted from variations in the experimental model employed.

There is still some confusion on the effects of splenectomy in man. Rowley (1950b) reported that splenectomized adults were unable to produce antibodies to a small intravenous dose of SRBC. Saslaw et al. (1959) and Myerson et al. (1957) failed to demonstrate impaired antibody titers in splenectomized patients immunized with tularemia vaccine and tetanus toxoid, respectively. These negative results are probably explicable in terms of the route of inoculation employed in these experiments, as is discussed below. There is ample evidence that splenectomized children are more susceptible to fulminating septicemias, and although it is feasible that impaired antibody formation may be a pathogenic factor in this syndrome, this has yet to be substantiated (Ellis and Smith, 1966).

Relatively little has been published on the relationship of age and spleen function. Phylogenetically, the thymus and spleen are the first two lymphoid organs to appear (Ellis and Smith, 1966). In the human infant the spleen is larger in relation to body weight, at birth, than at any other time of life (Ellis and Smith, 1966). With increasing age the size of the organ decreases (McCormick and Kashgarian, 1965). Makinodan and Peterson (1964) have reported that in mice, the primary antibody forming potential of the spleen decreased with age. They considered this to be due to a decrease in the actual numbers of

antibody-forming cells with increasing age.

The chemical nature of the antigen used for immunization probably affects the type of response seen in splenectomized animals. The data pertaining to this is confusing. Many investigators studied only the response to a single antigen (often erythrocyte stromata), and relatively few have compared the results of using different antigens. In practically all the studies with SRBC, splenectomized animals responded poorly or not at all (Rowley, 1950a; Rowley, 1950b; Taliaferro and Taliaferro, 1950; Draper and Süssdorf, 1957; Saslaw and Carlisle, 1964; Berglund, 1956; Adler, 1965; Motohashi, 1922).

Rowley (1950a) found that the response of the splenectomized rat to killed Friedlander's baccilli was similar to that against SRBC. Splenectomized monkeys responded normally to subcutaneous inoculation of typhoid or tularemia vaccine and intravenous administration of typhoid vaccine, although the antibody titers to SRBC were depressed (Saslaw and Carlisle, 1964). The systematic study of Fitch and Winebright (1962) and Winebright and Fitch (1962) deserves special comment. These authors found that splenectomy in the rat had a greater depressive effect on antibody titers to a particulate antigen (Salmonella typhosa flagella) than on the response to the soluble preparation, flagellin. The kinetics of the agglutinin response to these two antigens was quite different, both in intact and splenectomized rats. These observations raise interesting questions on the distribution and metabolism of different types of antigens, and also on the nature of the antibody molecules produced in response to them.

It appears that the effects of splenectomy are most marked when the immunizing dose of antigen is small. This was first demonstrated

by Motohashi (1922) and has been confirmed by Rowley (1950) and by Winebright and Fitch (1962). It has been suggested that the spleen is of especial importance in the phagocytosis and immune response to small doses of particulate antigens in the bloodstream (Ellis and Smith, 1966). With a large dose of antigen the spleen would therefore cease to be important, since the majority of antibody would be formed elsewhere (Motohashi, 1922). If these interpretations were correct, they would explain the susceptibility of children to fulminating septicemias following splenectomy (Ellis and Smith, 1966).

By virtue of its anatomical location, the immunological functions of the spleen are principally directed towards materials in the bloodstream. It is not surprising that some investigators have failed to demonstrate impaired antibody titers in asplenic animals, when immunization was by an extravascular route (Draper and Süssdorf, 1957; Myerson et al., 1957; Rowley, 1950; Saslaw et al., 1959). However, others have implicated the spleen in the production of antibodies following intraperitoneal or subcutaneous immunization (Adler, 1965; Rosenquist and Wolfe, 1962; Winebright and Fitch, 1962).

Effects of Splenectomy on the Kinetics of the Primary Response.

Taliaferro and Taliaferro (1950) found that splenectomy in the rabbit resulted in a delayed and depressed antibody response to SRBC. In a classical series of experiments they showed that the spleen was the primary seat of immunogenesis for a few days, after which it appeared to virtually 'shut down', so that the remainder of the response was due to extrasplenic antibody synthesis. This concept was supported by experiments which demonstrated that splenectomy on or after the day of peak hemolysin titer failed to affect the rate of

decay of antibody levels, indicating that the spleen was no longer a significant production site. Stelos and Taliaferro (1959) found that the rabbit produced the classical IgM-IgG sequence of immunoglobulins with SRBC. Thus the results of Taliaferro and Taliaferro (1950) are explicable in terms of a short period of IgM synthesis by the spleen, followed by IgG production by extrasplenic tissues. The results of Winebright and Fitch (1962), discussed earlier, lend support to this concept, especially in the light of the findings of Nossal, Ada and Austin (1964) that the rat produced both IgM and IgG antibodies to salmonella flagella, but only IgG antibodies to flagellin.

Fitch (1963) found that if rats were splenectomized up to 8 days after a single dose of Salmonella typhi flagella, the immune response was terminated, whereas splenectomy after 14 days had no effect. The early antibody peak, which he described, was probably due to macroglobulins. Cannon and Wissler (1965) detected the emigration of cells from the spleen of the rat during the course of primary immunization. These cells were morphologically similar to lymphocytes and were believed to colonize other lymphoid organs (Fitch and Wissler, 1965). This again suggests a central role for the spleen in early antibody, and hence by inference, IgM production.

Not all investigators have supported this postulate. Askonas, Humphrey and Porter (1956) indicated that the spleen formed antibodies later in the response (IgG?), and this was corroborated by Davidsohn, Lee and Zandrew (1964), since they found that while splenectomized rabbits appeared capable of producing gamma-1 (IgM?) antibodies normally, gamma-2 antibody production was impaired.

Summary and Conclusions.

Although the amount of literature on various aspects of spleen function is impressive, it is apparent that many gaps in our knowledge still remain.

The spleen forms one of the main lines of body defense against foreign materials that have gained access to the bloodstream. It appears to be involved in the early part of the immune response to such antigens, and hence must participate in the synthesis of macroglobulin antibodies, although the extent of its participation in this still needs clarification. At present there is no conclusive evidence that it possesses a population of cells which produce macroglobulins at a rate which is out of proportion to other antibody-forming tissues.

The Antibody Response in Bovine Anaplasmosis

Introduction.

Soon after Theiler (1910) characterized bovine anaplasmosis, both he and subsequent investigators showed conclusively that cattle which survived the primary infection became solidly immune. Furthermore they found that immunity was associated with latent infection, so that these animals became lifelong carriers (Mott, 1957).

Complement-Fixing and Agglutinating Antibodies.

In 1934 Rees and Mohler presented preliminary evidence that complement-fixing (CF) antibodies appeared during anaplasmosis (Rees and Mohler, 1934). However, it was not until a suitable antigen had been developed, that the CF test became of any practical value (Price, Brock and Miller, 1954). Within the past decade the CF test has become an integral part of the diagnosis and control of anaplasmosis in the

field.

Complement-fixing antibodies appear late in the prepatent period of the disease and rapidly reach a peak titer. Titers then slowly decline, but a positive reaction can be detected for long periods, usually for life. Although the presence of these antibodies demonstrates unequivocally that Anaplasma marginale is antigenic, and a positive titer is regarded to be synonomous with immunity, the biological significance of CF antibodies is unknown. They have been suggested to act as opsonins (Ristic and Sippel, 1958). Although this is an attractive suggestion, it has yet to be substantiated. Ristic (1962) has described the presence of agglutinating antibodies in infected animals, this being the basis of the capillary agglutination test for anaplasmosis. Again their significance as 'protective antibodies' remains undefined.

The Role of the Spleen in Anaplasmosis.

It has been known for some time that the spleen plays a central role in the development and maintenance of immunity to Anaplasma marginale. Splenectomy prior to infection increases the severity of the ensuing disease, and splenectomized cattle develop higher levels of parasitemia and a more severe anemia than unoperated controls (Murphy, 1964; Norman, 1966). The onset of the CF antibody response is significantly delayed in splenectomized calves (Norman, 1966).

Several authors have reported that splenectomy of the carrier animal led to an acute disease relapse. This was accompanied by a rapid fall in CF antibody titers (Norman, 1966; Ristic, 1960). Complement-fixing antibody titers rose again as marginal bodies appeared in the blood. This fall in CF antibodies may have resulted from the

general fall in gamma-globulins described by Dimopoullos, Foote and Schrader (1959) following splenectomy in uninfected calves.

The Kinetics of the Immunoglobulin Response in Anaplasmosis.

Dimopoullos, Schrader and Foote (1960) studied the serum protein changes in splenectomized calves with anaplasmosis. They found an early rise in alpha and beta-globulins coincident with the beginning of patent disease. During convalescence, alpha and beta-globulin levels returned to normal, and gamma-globulin levels increased. These findings were extended by Rogers and Dimopoullos (1962), who fractionated sera from infected animals by curtain electrophoresis. Acute phase sera contained CF activity in the alpha, beta and fast gamma-globulins. In early convalescence antibody activity was mainly associated with the gamma-globulins, with low concentrations in the beta-globulins. It was concluded that a shift in the distribution of antibodies occurred as the disease progressed, but the reasons for this were not explained.

Since the introduction of immunoelectrophoresis, the difficulties inherent in the interpretation of the results of classical zone electrophoresis have become very apparent. This was emphasized by Murphy (1964), who pointed out that zone electrophoresis per se cannot do more than associate antibody activity with poorly defined, heterogeneous groups of proteins.

Murphy (1964) studied the kinetics of the antibody response to Anaplasma marginale in terms of specific immunoglobulins. Early CF antibodies were exclusively in the IgM fraction, and after 4-5 days these were augmented by electrophoretically fast IgG antibodies (Murphy, Osebold and Aalund, 1966a). At no time was antibody activity found in the slow IgG fraction. In early convalescence the relative

proportions of IgM and IgG antibodies reached a plateau, where two-thirds were IgM and one-third IgG, and this situation persisted for many months. Murphy, Osebold and Aalund (1966a) failed to detect any differences between the kinetics of the immunoglobulin response in intact and splenectomized cattle. However, inspection of their data suggests that the time of onset of the CF antibody response was delayed in their splenectomized animals.

The macroglobulinemia which occurred was sufficiently marked to be detectable by immunoelectrophoresis of acute phase sera (Murphy, Osebold and Aalund, 1966b). Marked macroglobulinemia has also been found in malaria (Abele et al., 1964) and in trypanosomiasis (Masseyeff and Lamy, 1966; Mattern, Duret and Pautrizel, 1963). The reasons for this type of immunoglobulin response are still unclear, since little is known about the functions of macroglobulins in immunity.

The Relation of Age and Disease Intensity.

All ages of cattle are susceptible to infection with Anaplasma marginale. However, the calf under a year of age usually develops a mild, often subclinical, disease (Jones and Norman, 1962; Ristic, 1960; Roby, Gates and Mott, 1961). Under natural conditions the transfer of maternal antibodies to the calf no doubt complicates an accurate evaluation of innate resistance in this age group. Even in the absence of passive immunity, it has been established that the young calf is indeed more resistant to the effects of the organism than the adult (Jones et al., to be published).

Jones et al. (1967) have characterized the patterns of experimental anaplasmosis in cattle of different ages. The magnitude of parasitemia, and hence the severity of anemia, were found to be age-related,

being most severe in aged cows (over 10 years old). The three old cows that survived in these experiments had recrudescences of parasitemia in a cyclical pattern, similar to that described by Norman (1966) in splenectomized calves. Furthermore, there was a significant delay in the onset of the CF antibody response in the aged cows, compared with the calves (Jones et al., 1967). The high CF antibody titers that were observed in these cows were correlated with greater intensity of parasitemia, so that the serological response may well have been inferior to that of younger animals. These observations led Jones et al. (1967) to postulate that the old cow might be relatively 'hyposplenic', since its disease pattern resembled that of splenectomized calves. Suffice it to say that hyposplenism is a rather poorly defined entity, and an accurate assessment of hypofunction in a complex organ such as the spleen would be very difficult.

Immunization Studies.

For many years immunization of cattle against *Anaplasma marginale* basically relied on two methods. In some parts of the world cattle were inoculated with the relatively avirulent *Anaplasma centrale*, which produces cross-immunity. Alternatively, young calves were premunized with blood from carriers of *Anaplasma marginale*, thus inducing a mild disease with subsequent immunity (Brock, Kliewer and Pearson, 1965). Neither method is ideal, for obvious reasons.

Attempts to produce a useful vaccine have met with little success until recently. Now an inactivated vaccine is available, which contains Anaplasma antigens, but is incapable of producing infection (Brock, Kliewer and Pearson, 1965). This vaccine does not appear to prevent the establishment of natural infection, but it is effective in

eliminating fatalities, and it greatly reduces the incidence of clinical cases. The nature of the relative immune status produced by this vaccine is of considerable interest, since it suggests that there must be immune forces operative in anaplasmosis which are not dependent on the persistence of the live organism in the body.

CHAPTER III

MATERIALS AND METHODS

Experimental Design

Two groups of calves were used in these experiments: Group I consisted of five calves, which were sham-operated, and subsequently infected with Anaplasma marginale. Hematological and immunological observations were performed on these calves throughout the ensuing disease, extending into the convalescent period. Group II consisted of six calves, which were splenectomized prior to infection. The animals in this group were treated identically to those of Group I.

Experimental Animals

All the calves were castrated male Hereford or Hereford cross, approximately three months old at the time of purchase. They were kept in a dry lot, fed bluestem hay and cottonseed meal with a vitamin-mineral supplement.[1] The calves were periodically checked for internal and external parasites, and treatment for these was instituted as indicated. Freedom from prior exposure to anaplasmosis was ascertained at the time of purchase by means of the CF test.

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[1] Clovite, Fort Dodge Laboratories, Fort Dodge, Iowa.

Surgical Procedures

Group II calves were splenectomized via a left flank laparotomy. Group I calves were subjected to a sham operation, which entailed laparotomy and handling the spleen. All surgical procedures were performed under strict asepsis, using paravertebral anesthesia. Both groups of calves were given penicillin and streptomycin for five days postoperatively.

Exposure to *Anaplasma marginale*

Group I was infected 36 days after sham laparotomy, and Group II was infected 27 days after splenectomy. All of the calves were infected with blood from the same carrier animal. This blood was treated with neoarsphenamine (25 mg./10 ml. of blood) for 24 hours at 4°C. This prevented accidental transmission of *Eperythrozoon wenyoni*. Each calf received 1 ml. of treated blood subcutaneously over the shoulder.

Hematological Techniques

Blood samples were collected in the morning, both with and without ethylene diaminetetracetate (EDTA). Hematological observations were performed according to previously reported methods (Brock, 1958). Packed cell volume was determined by the microhematocrit method; hemoglobin by the acid hematin technique. Red blood cell (RBC) counts were made with an electronic grid counter[2] using a 1 in 100,000 dilution. Cover-slip smears were stained with Wright's stain, and the percentage

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[2]Coulter Counter, Model B, Coulter Electronics Co., Hialeah, Florida.

of parasitized erythrocytes (PPE) was calculated for a count of at least 1,000 RBC.

Complement-Fixation Test

On each day that the calves were bled, aliquots of serum were separated and frozen at -20°C. At the termination of the experiment, all the samples from any one animal were titrated for CF antibodies at the same time. The CF test was performed by a modification of the method of Price, Brock and Miller (1954). Samples were screened for positive reactions at a 1 in 10 dilution, and positive sera were subsequently titrated. A positive titer was regarded as the $\log_{10}$ of the denominator of the highest dilution giving a 4+ reaction (Norman, 1966).

Immunological Techniques

Gel Filtration.

A 7 x 70 cm. column was used in most of these experiments. The bed material, Sephadex G-200[3], was allowed to swell for at least three days in excess of Tris-buffer (0.1M Tris in 1.0M NaCl, pH 8.0). The column was packed by the method of Flodin (1961). It required some 70 gm. (dry weight) of Sephadex to fill the column. After packing, the column was equilibrated with several column-volumes of Tris-buffer.

Serum samples were mixed with sucrose (10% w/v) and applied with a syringe, with a length of polyethylene tubing attached to the needle. Even application was facilitated by the use of a sample applicator, consisting of a lucite tube, 2" long, with nylon net (400 mesh) across

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[3]Pharmacia Fine Chemicals, Piscataway, New Jersey.

its lower end. This rested on top of the gel bed. Sample volumes were kept within 1-4% of the bed volume. Elution was accomplished by gravity flow of Tris-buffer, and the flow rate was adjusted to remain less than 15 ml./sq. cm./hr. The hydrostatic pressure on the top of the gel was kept below 15 cm. of water throughout packing and elution. The flow rate did not decrease markedly over months of use.

Fractions of the eluate were collected with an automatic fraction-collector using a 5 ml. siphon. Fractions were assayed for protein content spectrophotometrically at 280 mu. The portions of the eluate which were retained were concentrated by dialysis against 20% polyvinylpyrrolidone (PVP) at 4°C.

Anion Exchange Chromatography.

Sera to be fractionated on DEAE-Sephadex[4] were dialyzed overnight against 0.02M phosphate buffer (PB), pH 8.0. About 10 gm. of DEAE-Sephadex was mixed with an excess of PB and allowed to swell at room temperature for several hours. The swollen gel was packed in a 2.5 x 50 cm. glass column and equilibrated with PB. Ten ml. of serum was applied to the column in the same fashion as described above. The column was eluted by gravity flow of buffer from a reservoir 30 inches above it. Elution was effected by means of a phosphate gradient, using a cylinder-cone (beaker-Erlenmeyer flask) reservoir system. The beaker contained 0.02M PB, pH 8.0, and the Erlenmeyer flask 0.37M monobasic sodium phosphate, pH 4.2. This system produces a concave phosphate gradient with increasing molarity and decreasing pH (Murphy, 1964). In some experiments, only the breakthrough peak of protein was eluted by

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[4]Pharmacia Fine Chemicals, Piscataway, New Jersey.

means of 0.02M PB. Five ml. fractions were collected and handled in analogous fashion to those from gel filtration experiments.

Preparative Zone Electrophoresis.

This technique employed the polyvinyl material 'Pevikon C-870'[5] (Müller-Eberhard, 1960). The apparatus was a modification of that described by Fahey and McLaughlin (1963). It consisted of a 37 x 12 x 2 cm. lucite tray and two buffer vessels with platinum wire electrodes, each one holding 4 l. of buffer.

About 230 gm. of Pevikon was thoroughly washed with distilled water, given a final wash in veronal buffer (VB) (ionic strength 0.075, pH 8.6), and finally suspended in a small amount of VB. The tray was fitted with wicks made from cotton towels and lined by parafilm. The slurry of Pevikon was poured into the tray, and excess moisture was allowed to drain off through the wicks, until a slot could be made in the block without it filling in.

Whole bovine serum, mixed with a trace of bromthymol blue, was applied to a 1 mm. slot cut in the block 12 cm. from the cathodal end. The sample (volume 5 ml.) was applied with a syringe and needle. The block was placed between the buffer vessels in the refrigerator, and the wicks were allowed to drop into the buffer. After 30 minutes the apparatus was connected to a power supply[6]. Electrophoresis was performed at a constant voltage of 350-400 v., until the blue-labeled albumin zone had reached the end of the block. This required some 18

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[5] Stockholms Superfosfat Fabriks A.-B., Stockholm, Sweden.

[6] Duostat, Beckman Instruments, Spinco Division, Palo Alto, California.

hours at 400 v. The block was then cut into 1 cm. transverse segments, each of which was eluted with about 7 ml. of phosphate-buffered saline (PBS) through separate sintered glass funnels into Erlenmeyer flasks attached to a vacuum. The fractions were centrifuged to remove any remaining Pevikon, and the protein concentration was read spectrophotometrically at 285 mu. Fractions were rapidly concentrated by packing dialysis bags in solid sucrose, the latter being removed by dialysis against Tris-buffer.

Immunoelectrophoresis and Immunodiffusion.

Immunoelectrophoresis (IE) was performed by a modification of the micromethod of Scheidegger (1955), using a commercially available apparatus[7]. One percent agar[8] was dissolved in veronal buffer (ionic strength 0.03, pH 8.6.) and poured onto 1 x 3 inch microscope slides. Standard two-well, single trench patterns were used. Electrophoresis was performed at 250 v. for 90 minutes. After appropriate antisera had been applied, the trays of slides were incubated for 18-24 hours at room temperature, in a humid box.

Double diffusion in gel by the method of Ouchterlony was performed as a micromethod, using parts of the IE apparatus. One percent agar was dissolved in phosphate-buffered saline (0.1M NaCl, 0.03M PO_4, pH 8.0), and the slides were incubated for 24-48 hours after sample application.

IE and gel diffusion patterns were recorded photographically be means of a 4 x 5 view camera and Polaroid Type 52 film. A simple dark-

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[7] Immunophor, LKB-Produkter, A.-B., Stockholm, Sweden.

[8] Ionagar, Oxo Ltd., London, England.

field illuminator was constructed from an x-ray viewer. Occasionally, the slides were washed for 24 hours in 2% saline, dried and stained with Amido-schwartz 10B.

Antiserum Production.

Healthy young rabbits were purchased locally, kept in individual cages and fed rabbit pellets and water ad lib. The following antisera were produced:

(1) anti-whole bovine serum

(2) anti-bovine IgG

(3) anti-bovine IgM

Antigen preparations were emulsified in an equal volume of complete Freund's adjuvant, and rabbits were immunized intramuscularly in the thigh. Immunization was usually repeated at weekly intervals. With purified immunoglobulin preparations, individual doses varied from 5-10 mg. of protein.

Rabbits were bled from the ear vein, and not more than 20 ml. of blood were taken at each bleeding. The serum was tested for specificity by IE and stored at -20°C.

Analytical Ultracentrifugation.[9]

Ultracentrifugation of purified immunoglobulins was performed in a Spinco Model 'E' ultracentrifuge[10]. The speed of centrifugation was 56,100 rpm. All samples were previously dialyzed against buffered saline (0.1M NaCl) pH 8.0. The observed sedimentation coefficients

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[9]Kindly performed by Mr. Arlan Richardson, Department of Chemistry, Oklahoma State University.

[10]Beckman Instruments, Spinco Division, Palo Alto, California.

were corrected to water at 20°C ($S_{20,w}$), and are expressed as Svedberg units ($S=10^{-13}$cm./sec./dyne/gm.).

Quantitative Radial Diffusion Test.

The levels of IgM and IgG in the sera of the experimental animals were assayed by means of an agar-antibody diffusion test, as described by Fahey and McKelvey (1965). In this test specific antiserum is incorporated in an agar layer on a plate. Standard preparations of the protein to be assayed, and unknown samples are placed in wells in the agar. As diffusion occurs, a precipitin ring forms around the well. The diameter of the ring is proportional to the concentration of the reactant being assayed.

A 2.5% solution of agar was prepared in PBS, pH 8.0 and allowed to equilibrate to 56°C in a waterbath. Antisera specific for IgG or IgM were diluted with PBS, an optimal dilution having been determined previously. In general, IgM antisera were diluted 1 in 11 and IgG antisera 1 in 9. Eight ml. aliquots of diluted antiserum were heated to 56°C. Clean 3 1/4 x 4 inch glass plates were coated around the edge with agar, and this was allowed to harden in an oven. The plates were placed in a lucite frame on a leveling table. Eight ml. of antiserum and 8 ml. of agar were mixed and rapidly poured onto the plate. Subsequently, 35 wells were punched in the agar layer with the aid of a template. These wells were 3 mm. in diameter and 10 mm. apart.

Antigen samples were applied with 2 x 75 mm. capillary tubes. The upper row of seven wells on each plate contained serial dilutions of a standard serum, with known IgM and IgG concentrations. This serum had been previously standardized against purified isolates of the two immunoglobulins. The protein content of the immunoglobulin preparations

was determined by a modification of the phenol reagent method, using crystalline bovine serum albumin to construct a standard curve (Lowry et al., 1951). The remainder of the wells were filled with the serum samples to be assayed. Samples for IgG determination were diluted 1 in 6 with PBS to reduce the size of the precipitin rings.

The plates were incubated in closed humid boxes for 18-24 hours at 4°C. They were then washed for 24 hours in several changes of 2% saline, dried under moist filter paper, stained with Amido-schwartz 10B, and rinsed with acidified methanol.

The diameter of the precipitin rings was measured to the nearest 0.1 mm. with the aid of a magnified scale[11]. A standard curve was prepared for each plate by plotting the immunoglobulin concentration of the standard serum samples (in mg/ml) against the size of the precipitin ring (in mm.) on semi-logarithmic graph paper. The resulting relationship was linear, with slight deviations from linearity at very low and very high protein concentrations. The concentration of the immunoglobulin in the unknown sera could thus be read directly from the curve. Each unknown serum was assayed in triplicate, and a mean value calculated.

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[11]Bausch and Lomb, Rochester, New York.

CHAPTER IV

RESULTS

List of Abbreviations

The following abbreviations will be used throughout the discussion and in the legends of the figures:

AIgG - rabbit anti-bovine IgG antiserum.

AIgM - rabbit anti-bovine IgM antiserum.

AWS - rabbit anti-whole bovine serum.

CF - complement-fixing.

DEAE-DEAE-Sephadex (e.g. "DEAE Pk I" - protein from the first peak of DEAE-Sephadex chromatography of serum).

G200 - Sephadex G200 (e.g. "G200 Pk I" - protein from the first peak of gel filtration chromatography).

IE - immunoelectrophoresis.

IgG - immunoglobulin-G.

IgM - immunoglobulin-M (also macroglobulin).

PBS - phosphate-buffered saline.

PCV - packed cell volume (hematocrit).

Pev - Pevikon electrophoresis (e.g. "Pev 1-14" - fractions 1 to 14 from the cathodal end of a Pevikon block).

PPE - percentage of erythrocytes parasitized with Anaplasma marginale.

RBC - erythrocyte(s).

WS - whole bovine serum.

Isolation of IgM

Bovine IgM was prepared by a two-stage procedure. Whole serum was separated by Pevikon electrophoresis, and the slow half of the beta-gamma globulin peak was harvested. (Figure 1,A). The Pevikon fractions were pooled in such a fashion that the pool contained little protein migrating to the cathodal side of the origin on IE analysis. (Figure 2,a). Three to four of such pools were applied to a column of Sephadex G200. The resulting chromatogram consisted of two peaks (Fahey and McLaughlin, 1963). (Figure 1,B). The first peak produced a single precipitin line on IE, which migrated cathodally and appeared to emerge from the very edge of the antigen well. (Figure 2,b). This line was analogous to that described by Murphy (1964) for bovine IgM. Upon analytical ultracentrifugation this protein produced a peak migrating at a rate of 18.2S. (Figure 3).

Rabbits were immunized with the first half of the first peak of the gel filtration runs. Possible contamination with high molecular-weight complexes of other proteins (e.g. IgA) was thereby minimized. Although this protein appeared to be homogeneous as judged by IE, gel diffusion and ultracentrifugal analysis, rabbits immunized with the isolate produced antisera which reacted with IgG and an unidentified alpha globulin. (Figure 2,c). The presence of antibodies to IgG is not surprising, since the immunoglobulins of other species carry common antigenic determinants on their L polypeptide chains (Fahey, 1965). The alpha globulin must have been present as a trace contaminant in the IgM isolate, but in sufficient concentration to be immuno-

genic in the rabbit.

These antisera were rendered specific for IgM by absorption with 1) peak II protein from gel filtration of whole serum (IgG) and 2) an alpha-globulin preparation from Pevikon electrophoresis. Absorption was repeated with very small amounts of these antigens until no further precipitate was obtained. Gel diffusion analysis of an absorbed AIgM sample is shown in Figure 4. The precipitin line with whole serum produced a reaction of identity with that of purified IgM. These antisera produced well defined precipitin rings in the radial diffusion test for IgM. (Figure 9).

Isolation of IgG

Immunoglobulin-G was isolated by anion-exchange chromatography of whole bovine serum on DEAE-Sephadex (Murphy, 1964). The first peak of the chromatogram, which consisted of protein that did not become absorbed to the anion-exchanger, was pooled and concentrated. (Figure 5). On IE, it consisted of a pure isolate of electrophoretically slow IgG. (Figure 6,a). On analytical ultracentrifugation, it produced a single peak, with a sedimentation coefficient of 6.3S. (Figure 7). When rabbits were immunized with this preparation, the antisera reacted against both fast and slow IgG, indicating that these fractions contained common antigenic determinants. (Figure 6,b). Figure 8 shows gel diffusion analysis of AIgG reacted against whole serum samples, which were diluted 1 in 6 with PBS. Duplication of the precipitin arc occurred with all these sera. This has been described by Murphy (1964) with bovine IgG. He associated the phenomenon with the spur on the precipitin arc of IgG on IE. (Figure 6,b). In the present study,

these antisera produced double precipitin rings in the radial diffusion test for IgG. (Figure 9). The diameter of the outer ring was used to compute IgG levels. The reasons for the duplication of the precipitin line were not determined. Edelman et al. (1960) related doubling of the arc of human IgG with the presence of distinct antigenic determinants on the S and F papain fragments of the molecule. Anti-IgG antisera were absorbed repeatedly with peak I protein from Sephadex G200 gel filtration of whole serum, prior to use in IgG determinations.

Characteristics of Anaplasmosis in Groups I and II

In the following discussion, observations have been expressed in relation to Day 0, which is defined as the first day of patent disease, when 1% of the RBC were infected with Anaplasma marginale.

The prepatent period in both groups was about 30 days. After the onset of patent infection, levels of parasitemia increased rapidly, reaching a peak around Day +7. The splenectomized calves (Group II) reached much higher levels of parasitemia than those in Group I ($p=0.001$). The intense parasitemia was accompanied by a correspondingly more severe anemic crisis in Group II ($p=0.01$), and all six calves in this group died. The characteristics of the disease in the two groups are summarized in Figure 10 and in Table I. Group I calves recovered rapidly. The PPE returned to 1% by Day +13, although all the calves had persisting low levels of parasitemia for many days.

Calf #159 was about 3 months old at the time it was splenectomized, and it was not infected until 4 1/2 months after splenectomy. This calf survived the acute disease, in spite of a severe anemic crisis. Subsequently, it developed recrudescences of parasitemia. (Figure 11).

These are characteristic of the splenectomized animal with anaplasmosis (Norman, 1966). Since this calf was splenectomized earlier than those in Group II, its immunoglobulin response will be considered separately.

Pretreatment Immunoglobulin Levels in Groups I and II

Normal levels for IgG and IgM for each of the experimental animals have been tabulated in Tables II and III. The overall mean value for IgM was 1.72±0.64 mg./ml. and 19.8±4.11 mg./ml. for IgG. Immunoglobulin levels for the preinfection period were compared with those of the preoperative period (Group I was infected 36 days after sham laparotomy). IgM levels were significantly depressed following splenectomy in Group II ($p=0.05$). (Table II). No such difference was seen with IgG values. (Table III). IgM and IgG levels in Group I were similar before and after sham laparotomy.

Coefficients of variability (CV) were calculated for replicates of IgM and IgG determinations on a number of sera to provide an estimate of the technique error of the radial diffusion test. The CV for IgM determinations was 10% and for IgG 13%.

Since preinfection immunoglobulin values varied from calf to calf, these differences were obviated by expressing the immunoglobulin response as percentage deviations from the preinfection mean for each animal. Each preinfection mean was derived from the values on five separate days, each sample being assayed in triplicate on the radial diffusion test.

The IgM Response During Anaplasmosis

In Group I the preinfection IgM level was 1.65 mg./ml. (range 1.3

to 2.2), and in Group II 0.99 mg./ml. (range 0.77 to 1.58). The difference between these two means was significant (p=0.01). During the disease phases, sera from every third day were assayed for IgM and IgG, and the deviations from the preinfection means computed.

IgM levels fell slightly during the prepatent period in 5 of the 6 splenectomized calves and in 2 of the 5 intact calves. (Figure 12,A; Tables VI and VIII). Since the standard deviation of preinfection IgM levels was about 20% of the mean, a significant change was regarded as a persistent increase of over 20% above the mean. On this basis, in Group I IgM levels started to increase on Day -7 (range -8 to -3) and rapidly reached a peak on Day +4 (range 0 to +9). This peak ranged from 121 to 264% (mean 198%) above each calf's preinfection mean. (Table IV). Subsequently, IgM levels fell slightly, and in 4 of the calves a second smaller peak occurred 12 to 21 days after the first. (Table VI). This biphasic pattern has been obscured in the construction of the group mean in Figure 12,A. Levels of IgM then fell, at first rapidly, and then more slowly. By the termination of the experiment (Day +80), 3 of the calves still showed elevated IgM levels. (Table IV).

In Group II the onset of the IgM response was delayed. (Figure 12,A; Table VIII). Using the same criteria as before, significant elevations in IgM levels did not occur until around Day) (range -4 to +6). This delay was significant (p=0.01). However, the rate of increase of IgM values was similar in both groups (Figure 12,A), and some of the splenectomized animals reached quite high levels of this immunoglobulin before death. Direct comparison of peak values in the two groups was impossible, since it was not certain whether Group II calves had reached the zenith of their response before they died. The maximal IgM levels

observed in Group II ranged from 41 to 340% (mean 165%) above their preinfection means. The characteristics of the IgM response have been summarized in Figure 12,A and in Tables IV, VI and VIII.

Calf #159 appeared to show a slightly delayed IgM response. (Figure 13,A). In this splenectomized calf, IgM levels increased on Day -3, and reached a peak of +291% on Day +14, 4 days after its peak of parasitemia. Levels then fell quite rapidly but remained elevated above normal. There was a second smaller peak on Day +61, three days before the second peak of parasitemia.

The IgG Response During Anaplasmosis

The criteria used to determine a significant increase in IgG levels were the same as those for IgM.

Five of the 6 calves in Group II showed a slight fall in IgG during the prepatent period. This was not seen in Group I, except in one calf. (Figure 12,B; Tables VII and IX).

In Group I an increase in IgG levels occurred 11.5 days after the initial increase in IgM (range 6 to 18 days). This was followed by a small peak on Day +15 (range +12 to +18) and a larger peak on Day +40 (range +36 to +52). Figure 12,B; Tables IV and VII). The peak of the IgG response was comparatively small, ranging from 41 to 117% (mean 64%) above the preinfection means. The levels of IgG then fell quite rapidly, the duration of the response being 55.6 days (range 41 to 68 days).

In Group II IgG levels were depressed throughout the prepatent period. (Figure 12,B). Four of the 6 calves showed a small, though insignificant, increase in IgG just before death. (Table IX).

In calf #159 IgG levels remained below normal until Day +17, when

they slowly rose to reach a peak of 28% above normal on Day +52. (Figure 13,A).

Relationship of CF Antibody Titers with the Immunoglobulin Response

In Group I, CF antibodies appeared on Day -7.8 (range -7 to -10) and reached a peak 10.6 days later (range 4 to 17). (Table V). Peak titers ranged from 3.1 to 4.3 (mean 3.8)[1]. Titers then reached a plateau until Day +50, when they fell slowly. (Figure 14).

The time of appearance of CF antibodies (Day -7.8) and the time of the initial IgM response (Day -6.6) were similar. However, the correlation between these two values was poor (r=0.5). This disparity appeared to be due to two calves, in which CF antibody titers preceded the onset of the IgM response by several days.

In contrast, there was good correlation between the day of peak CF antibody titers (Day +3.4) and the day of maximal IgM levels (Day +4.8). The correlation coefficient (r=0.97) was significant at the 5% level. There appeared to be little relationship between the magnitude of peak CF antibody titers and peak IgM values.

Comparison of the mean curves of IgM, IgG (Figure 12,A and B) and CF antibodies (Figure 14) for Group I, suggested that the plateau region of the CF antibody response corresponded temporally with the plateau of the IgM response. Furthermore, the slight fall in CF antibody titers, commencing around Day +20, corresponded to the time when IgM levels were decreasing quite rapidly, and IgG levels were rising to a

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[1]CF antibody titers are expressed as the Log_{10} of the denominator of the highest dilution giving a 4+ reaction.

peak.

In Group II, the onset of CF antibody response was delayed. (Figure 14; Table V). The difference between Groups I and II was highly significant ($p=0.01$). Again, although both the initial CF antibody and IgM response in Group II commenced around Day 0, the correlation between these two variables was poor.

In calf #159, CF antibodies appeared on Day -4, one day before the onset of the IgM response. (Figure 13,B). This calf did not develop very high titers, in spite of quite intense parasitemia. After Day +12 its titers fell quite rapidly and remained at low levels until the end of the experiment. The plateau of CF antibody activity corresponded with the peak of IgM, but the latter persisted longer than the former. Although the recrudescence of parasitemia after Day +60 was accompanied by a transient IgM response, no change in CF antibody titers was detectable.

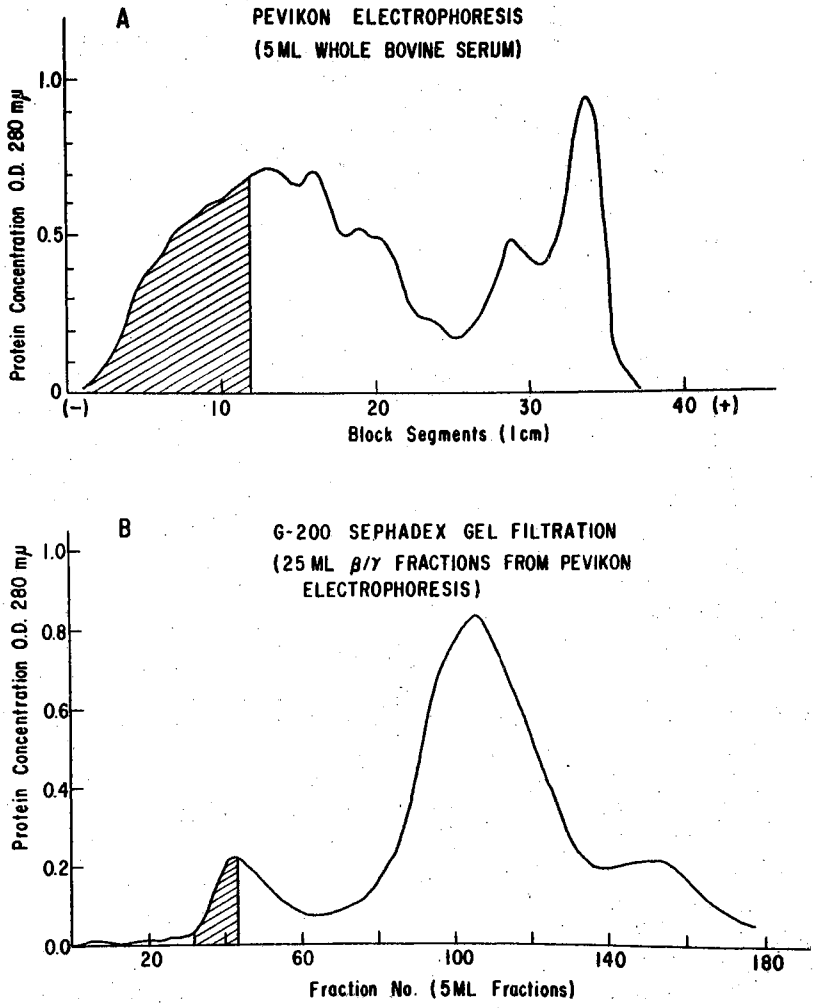

Figure 1. Isolation of bovine IgM. A) Electropherogram of Pevikon electrophoresis of whole serum; B) Chromatogram of Sephadex G200 gel filtration of pooled beta-gamma globulin fractions from (a). Shaded areas represent pooled fractions.

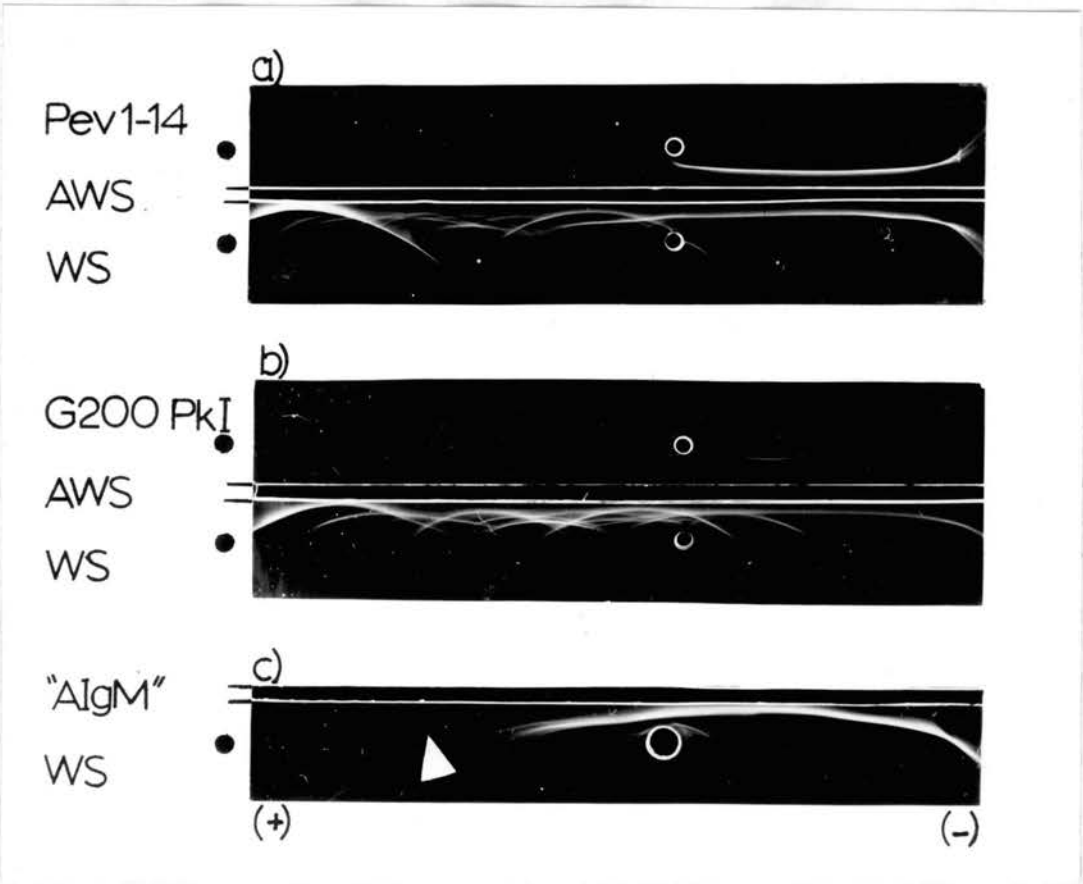

Figure 2. Immunoelectrophoretic analysis of steps in the isolation of IgM: a) beta-gamma globulin fractions of Pevikon electrophoresis; b) peak I from G200 gel filtration of (a); and c) antiserum prepared against (b). A faint arc is visible in the alpha-globulin region (arrow).

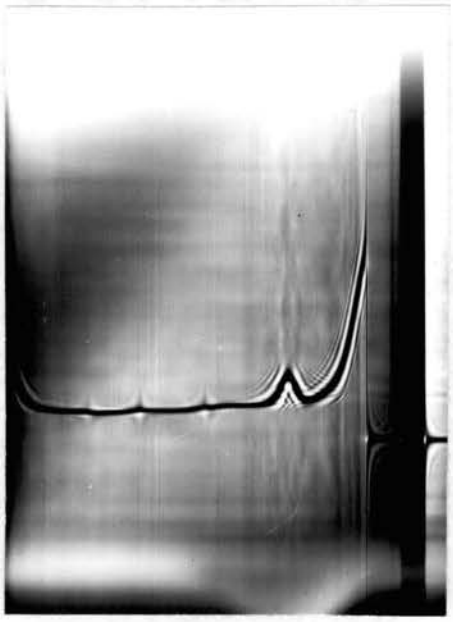

Figure 3. Ultracentrifugal analysis of bovine IgM. Speed of centrifugation - 56,100 rpm. Solvent - 0.1M NaCl. Time - 4 minutes after reaching speed.

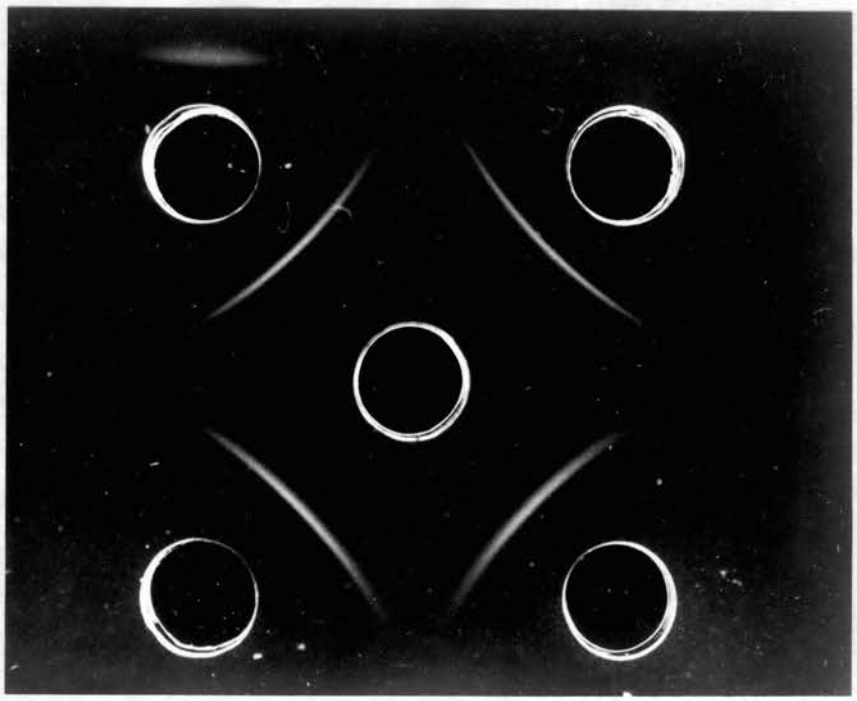

Figure 4. Double diffusion analysis of rabbit anti-bovine IgM. The upper left and lower right wells contained whole serum; the upper right and lower left wells, purified IgM. The center well contained absorbed AIgM.

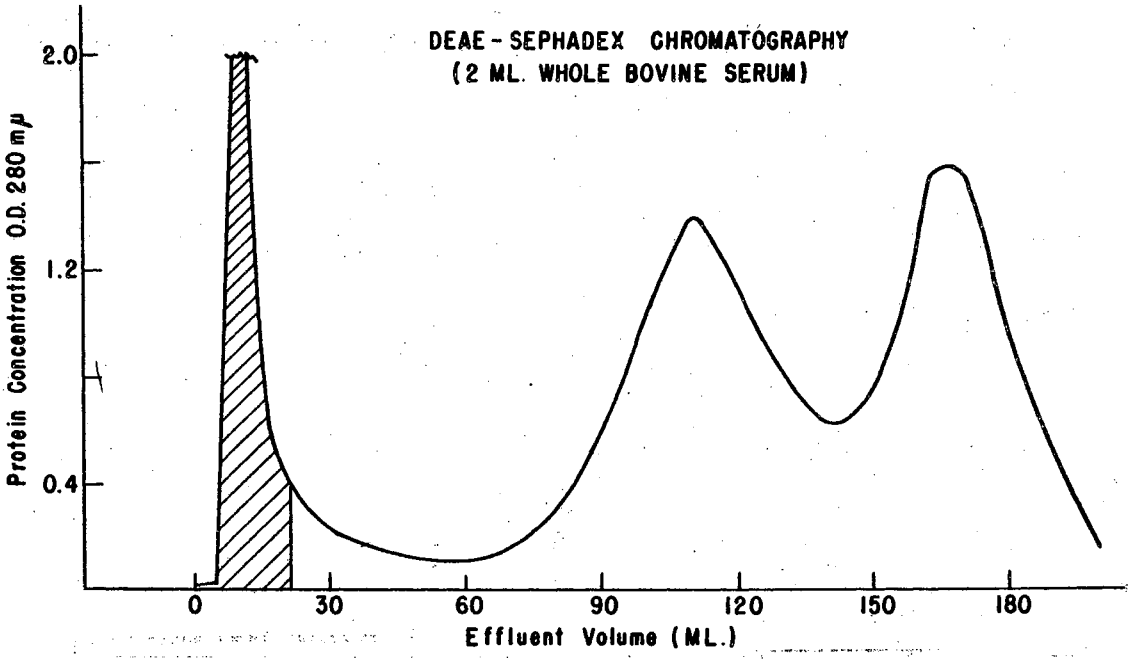

Figure 5. DEAE-Sephadex chromatography of whole bovine serum. The column was eluted by a gradient of phosphate buffer (0.02M to 0.37M). The shaded area represents pooled fractions.

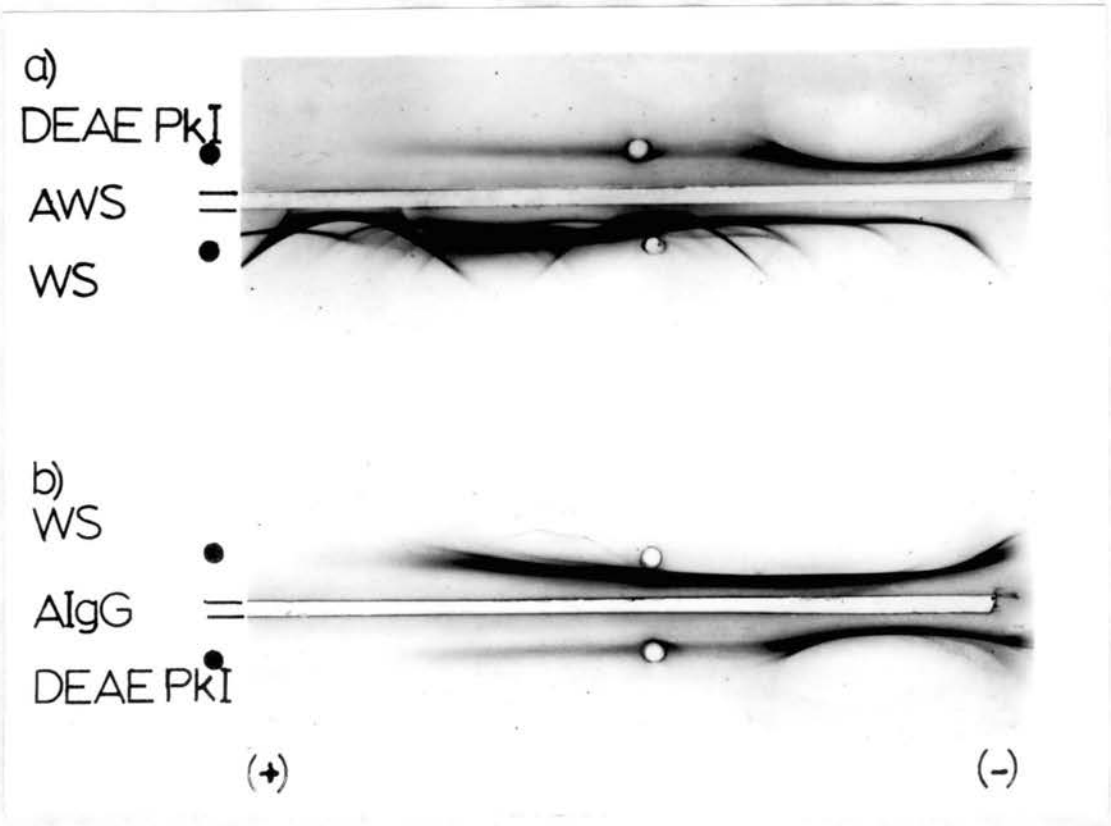

Figure 6. Immunoelectrophoretic analysis of IgG: a) Peak I protein from DEAE-Sephadex chromatography of whole serum; b) pattern of antiserum prepared against (a).

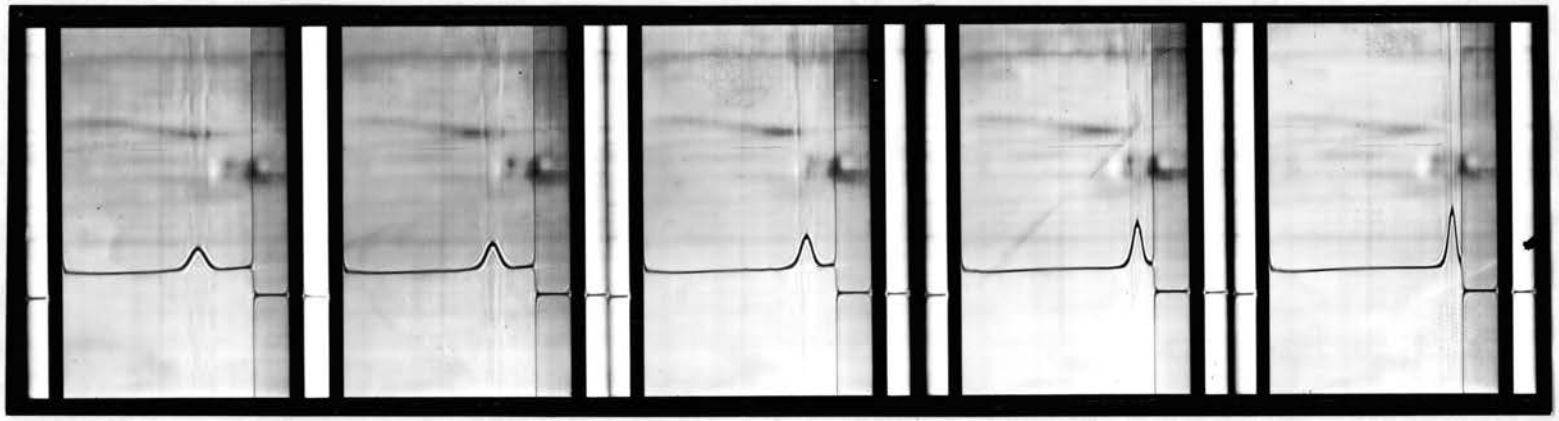

Figure 7. Ultracentrifugal analysis of bovine IgG. Speed of centrifugation - 56,100 rpm. Solvent - 0.1M NaCl. Time of photographs (from right to left) - 5, 10, 20, 30, 40 minutes after reaching speed.

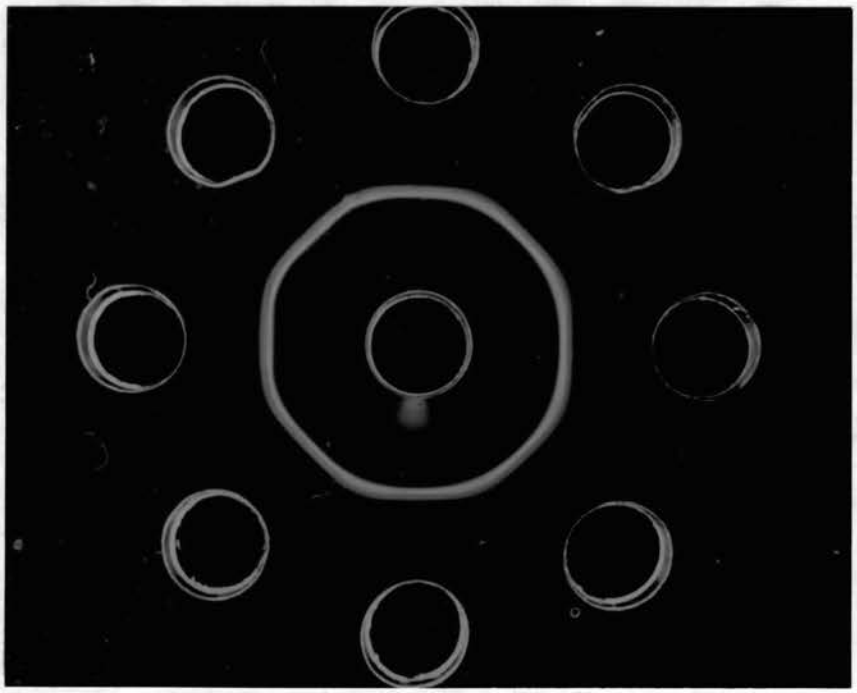

Figure 8. Double diffusion analysis of anti-bovine IgG. The outer wells contained different bovine sera, which were diluted 1 in 6 with saline. The central well contained anti-IgG antiserum.

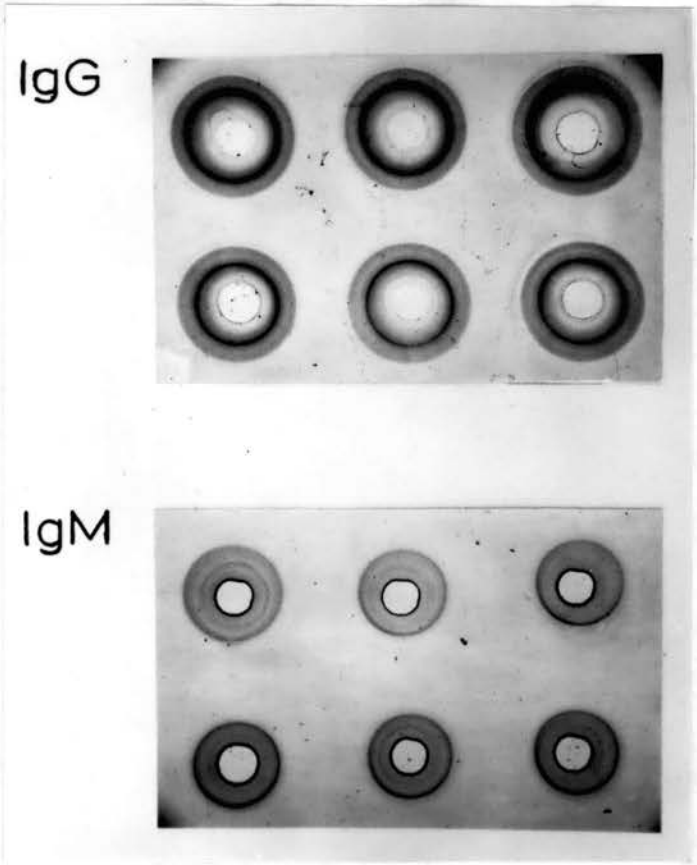

Figure 9. Representative examples of precipitin rings in the radial diffusion test for IgG and IgM. Stained with Amidoschwartz 10B.

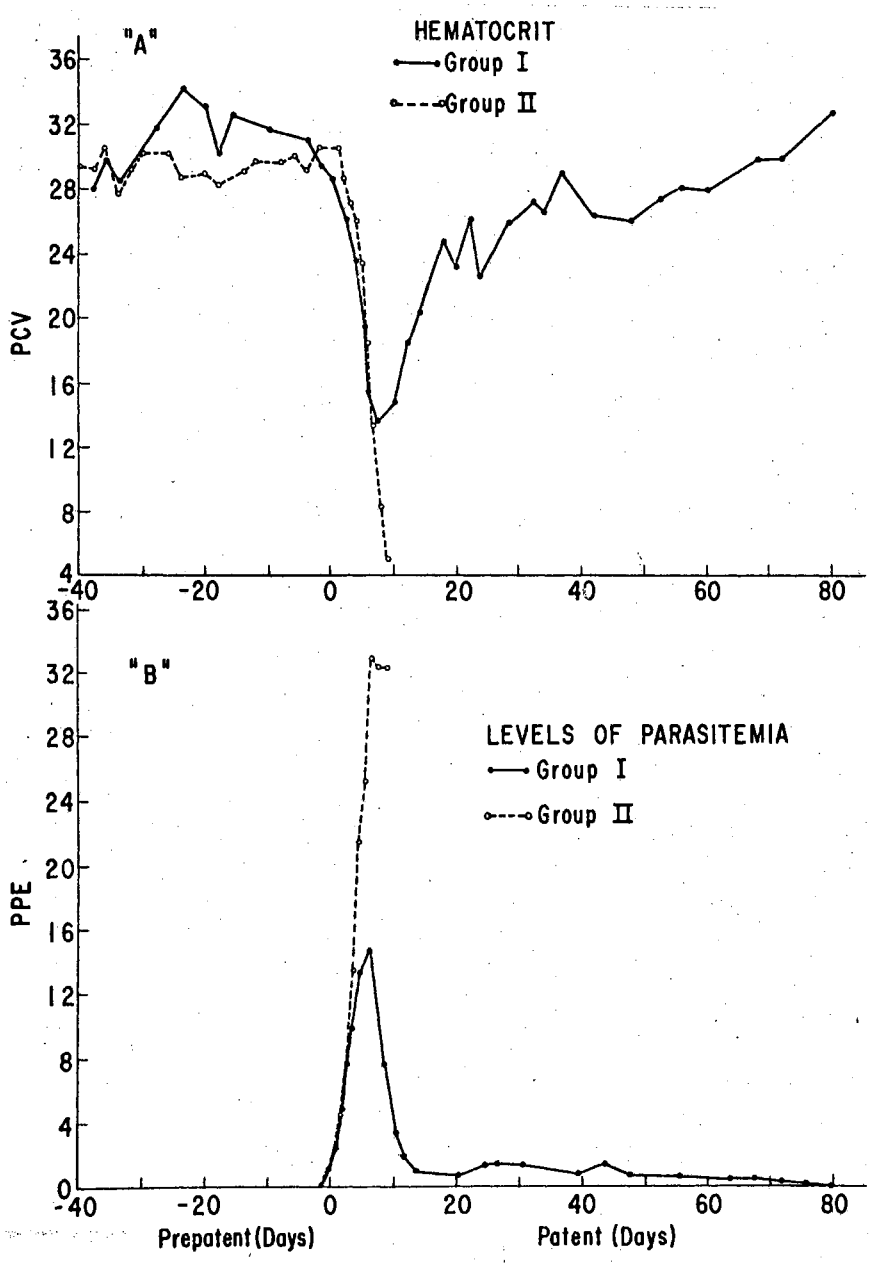

Figure 10. The characteristics of anaplasmosis in Groups I and II. A) Hematocrit. B) Levels of parasitemia.

TABLE I

CHARACTERISTICS OF EXPERIMENTAL ANAPLASMOSIS IN GROUPS I AND II

	Group I	Group II	t-test
	(Mean and Range)	(Mean and Range)	
Prepatent period (days)	30.4 (28-31)	28.2 (25-31)	n.s.d.
Day of maximal parasitemia	+6.4 (+4-+7)	+7.3 (+5-+9)	n.s.d.
Maximal parasitemia (%)	16.3 (12.5-19.2)	34.5 (30.3-38.5)	p=0.001
Duration of parasitemia* (days)	13.2 (13-14)	----	-----
Day of minimal PCV	+7.8 (+7-+9)	+8.2 (+6-+9)	n.s.d.
Minimal PCV (%)	12.4 (9.5-15.5)	7.2 (5.5-9.5)	p=0.01
Mortality (%)	0	100	-----

All times have been expressed in relation to Day 0, the first day of patent disease.

*Duration of primary parasitemia-time from Day 0 for the PPE to return to 1%.

n.s.d. - no significant difference.

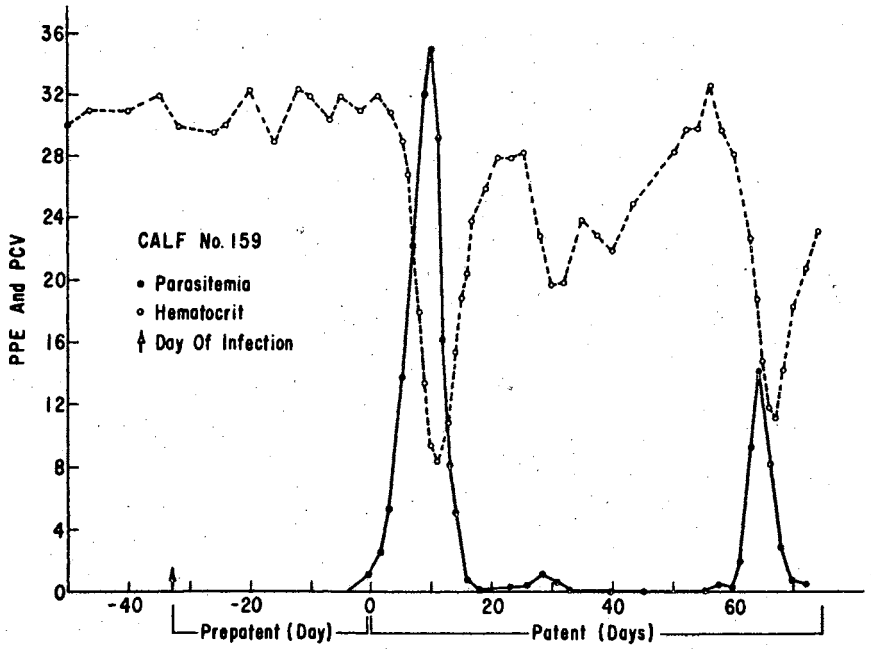

Figure 11. The characteristics of anaplasmosis in Calf #159. (Hematocrit and parasitemia).

TABLE II

COMPARISON OF IgM LEVELS (mg./ml.) IN GROUPS I AND II
IN THE PREOPERATIVE AND PREINFECTION PERIODS

Group I						
Animal number	153	155	158	160	163	$\bar{x}$
Prelaparotomy	1.49	(3.53)	1.31	1.98	1.82	1.65
Preinfection	1.74	(2.28)	1.25	1.60	2.16	1.68

Group I calves were infected 36 days after laparotomy. Preinfection values represent the means of 5 days immediately prior to infection.

Calf #155 had abnormally high IgM levels; its data has been ommitted from the statistical analysis.

$\bar{x}$ - mean.

Group II							
Animal number	164	165	166	167	168	169	$\bar{x}$
Presplenectomy	1.64	1.84	1.47	1.13	1.27	1.48	1.47*
Preinfection	0.77	1.58	0.93	0.95	0.82	0.92	0.99*

Group II calves were infected 27 days after splenectomy.

*The difference between these two means was highly significant, as judged by the Student's t-test.

$\bar{x}$ - mean.

TABLE III

COMPARISON OF IgG LEVELS (mg./ml.) IN GROUPS I AND II IN THE PREOPERATIVE AND PREINFECTION PERIODS

Group I						
Animal number	153	155	158	160	163	$\bar{x}$
Prelaparotomy	19.6	13.2	18.3	22.0	18.8	18.4
Preinfection	13.2	18.4	17.4	20.6	14.7	16.9

Group I calves were infected 36 days after laparotomy. Preinfection values represent IgG levels on several days immediately prior to infection.

$\bar{x}$ - mean.

Group II							
Animal number	164	165	166	167	168	169	$\bar{x}$
Presplenectomy	14.7	20.4	28.5	22.9	21.5	18.2	21.0
Preinfection	19.4	19.5	30.3	20.9	19.3	16.5	21.0

Group II calves were infected 27 days after splenectomy.

$\bar{x}$ - mean.

There were no significant differences between intra- or inter group means, as judged by the t-test.

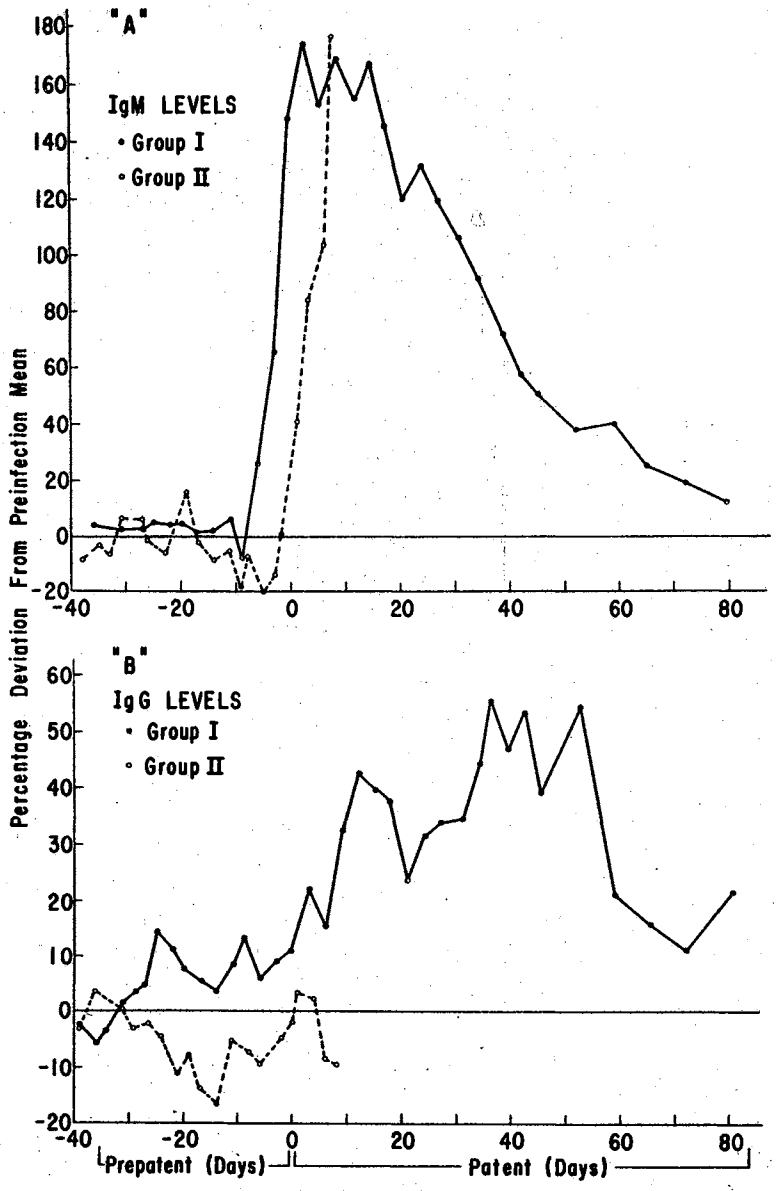

Figure 12. The immunoglobulin response in Groups I and II during anaplasmosis. A) IgM B) IgG. Immunoglobulin levels are expressed as percentage deviations from the preinfection mean of the group. Day 0 is the first day of patent disease.

TABLE IV

CHARACTERISTICS OF THE IMMUNOGLOBULIN RESPONSE IN GROUP I CALVES

Calf number	153	155	158	160	163	$\bar{x}$
Day of first IgM response*	-7	-7	-8	-8	-3	-6.6
Day of peak IgM response	0	+3	+3	+9	+9	+4.8
Peak IgM level (% increase)	121	230	158	264	217	198
Duration of IgM response (days)	46	over 87	over 88	over 88	55	
Day of first IgG response*	-4	+11	+3		+3	+1.4
Day of peak IgG response	+42	+36	+36	+52	+36	+40
Peak IgG level (% increase)	95	33	42	41	117	64.2
Duration of IgG response (days)	55	51	49		68	55.6

All times have been expressed in relation to Day 0; the first day of patent disease.

*A significant response was taken as a persistent increase of more than 1 standard deviation of the preinfection mean (i.e. over 20%). The duration of the response was calculated on the same basis - the time taken to return to 20% above the preinfection mean.

$\bar{x}$ - mean.

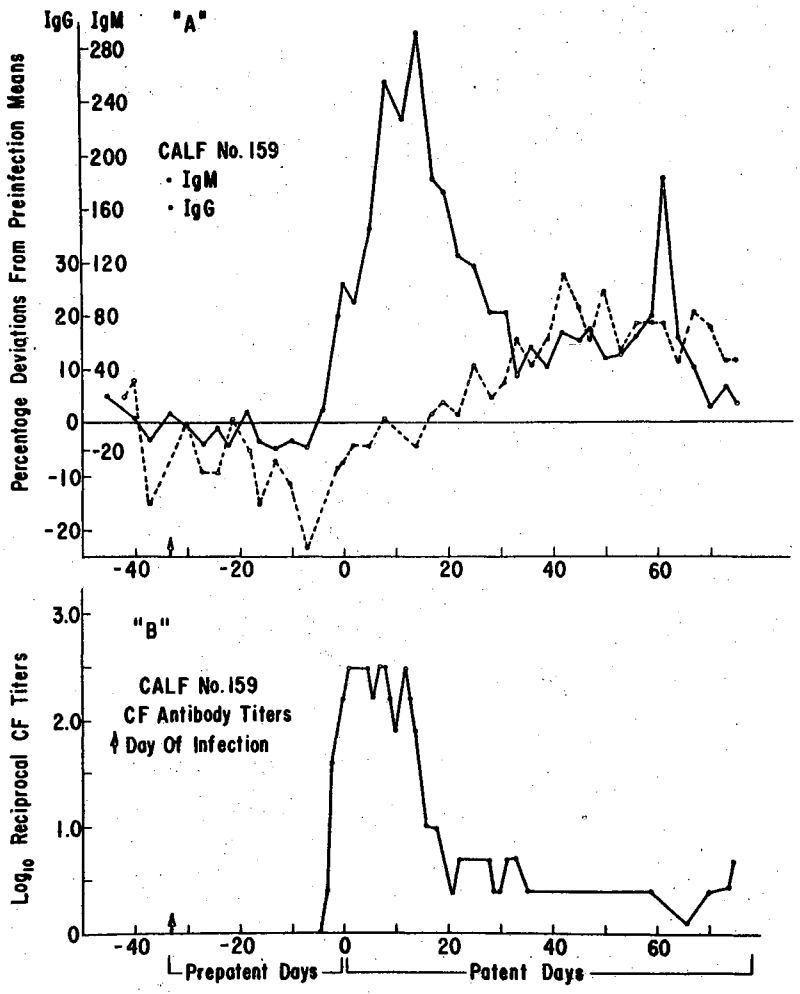

Figure 13. The immunoglobulin response in Calf #159 during anaplasmosis. A) IgM and IgG, B) CF antibody titers. Immunoglobulin levels are expressed as percentage deviations from the preinfection mean.

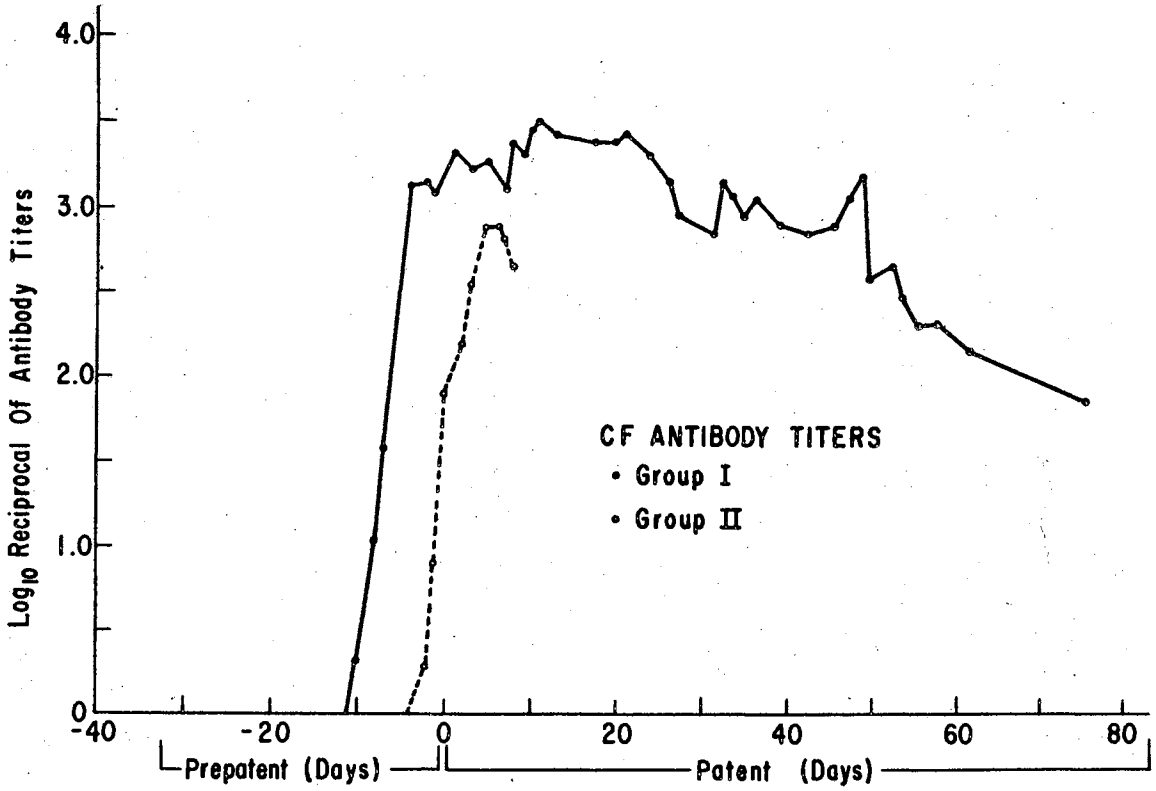

Figure 14. The complement-fixing antibody response in Groups I and II during anaplasmosis. Day 0 is the first day of patent disease.

TABLE V

CHARACTERISTICS OF THE COMPLEMENT-FIXING ANTIBODY RESPONSE IN GROUPS I AND II

Group I						
Animal number	153	155	158	160	163	$\bar{x}$
Day initial CF response	-10	-7	-7	-8	-7	-7.8
Day maximal CF response	-6	0	+5	+8	+10	+3.4
Maximal CF titer*	3.7	3.1	3.7	4.3	4.3	3.8

*CF titers are expressed as $\log_{10}$ of the denominator of the highest dilution giving a 4+ reaction.
$\bar{x}$ - mean.

Group II							
Animal number	164	165	166	167	168	169	$\bar{x}$
Day initial CF response	-1	-2	-2	-2	-3	-1	-0.8
Peak CF titer observed*	2.5	3.7	2.8	3.4	3.1	3.4	3.15

*Since all the calves in this group died, it is doubtful if these values represent the maximal response that would have been observed if they had survived.
$\bar{x}$ - mean.

Times are expressed in relation to Day 0; the first day of patent disease.

TABLE VI

SERUM IgM LEVELS (mg./ml.) DURING ANAPLASMOSIS IN GROUP I CALVES

Day	Calf Number 153	155	158	160	163	$\bar{x}$
-39	2.10	2.15	1.46	1.28	---	2.19
-36	1.86	1.41	1.18	1.41	3.13	1.79
-34	1.53	1.23	1.16	1.51	2.3	1.54
-31	1.5	1.1	1.2	2.2	2.0	1.6
-29	1.5	1.3	1.4	2.1	1.8	1.6
-27	1.6	1.1	1.5	2.2	1.6	1.6
-25	1.7	1.3	1.6	2.1	1.5	1.6
-22	1.5	1.3	1.5	1.9	1.9	1.6
-20	1.5	1.5	1.5	1.9	1.8	1.6
-17	1.4	1.5	1.4	2.0	1.6	1.6
-14	1.5	1.4	1.4	1.9	1.7	1.6
-11	1.7	1.3	1.6	1.8	2.0	1.7
-9	1.5	1.4	1.3	1.7	1.7	1.5
-6	2.3	1.7	2.0	2.2	1.6	1.9
-3	2.7	2.3	2.2	3.2	2.5	2.6
0	3.9*	3.6	2.7	4.3	1.7	3.2
+3	3.3	4.1*	3.2*	4.7	6.6	4.4
+6	3.2	3.9	2.4	5.0	5.8	4.1
+9	3.1	3.8	2.3	5.8*	6.9*	4.4*
+12	2.7	3.4	2.6	5.4	6.5	4.1
+15	3.2	3.5	3.1	5.6	5.9	4.3
+18	2.9	3.5	2.8	5.0	5.2	3.9
+21	2.6	3.6	---	5.7	4.4	4.1
+24	2.3	3.8	2.5	5.7	3.6	3.6
+27	2.3	3.5	2.6	5.3	3.2	3.4
+31	2.6	3.0	2.5	4.5	3.3	3.2
+34	2.4	2.5	2.4	4.5	3.3	3.0
+36	2.5	2.6	2.1	4.5	3.2	3.0
+39	2.1	2.3	2.2	3.9	3.0	2.7
+42	1.6	2.3	1.9	3.9	2.5	2.5
+45	1.5	2.0	2.1	3.3	2.9	2.3
+52	1.3	---	2.0	3.1	2.7	2.3
+59	1.7	1.6	1.7	3.7	2.0	2.1
+65	1.3	1.8	2.2	2.8	1.7	1.9
+72	1.4	1.8	2.0	2.2	2.1	1.9
+80	1.4	1.7	1.9	2.2	1.6	1.8

All observations have been expressed in relation to Day 0; the first day of patent disease.

*Peak IgM level observed for each calf.

$\bar{x}$ - mean.

TABLE VII

SERUM IgG LEVELS (mg./ml.) DURING ANAPLASMOSIS IN GROUP I CALVES

	Calf Number					
Day	153	155	158	160	163	$\bar{x}$
-39	----	19.8	18.0	21.2	9.8	17.2
-36	13.6	19.2	15.8	20.2	9.0	15.6
-34	13.0	19.2	16.0	20.2	11.6	16.0
-31	13.4	15.2	18.4	20.0	15.0	16.4
-29	12.8	18.8	18.4	21.2	14.2	17.1
-27	14.2	18.0	18.0	19.6	15.3	17.0
-25	15.3	16.6	20.2	20.6	18.6	18.3
-22	15.0	17.2	18.8	22.6	16.2	18.0
-20	13.8	12.8	18.8	21.8	18.6	17.2
-17	15.6	18.8	17.8	21.4	15.6	17.8
-14	13.0	14.8	16.8	21.0	17.4	16.6
-11	15.0	17.2	17.8	21.4	16.2	17.5
-9	15.8	15.6	19.4	19.8	15.8	17.3
-6	14.2	17.4	16.8	18.8	15.8	16.6
-3	18.2	15.2	17.0	19.8	16.2	17.3
0	16.2	16.2	17.4	19.6	18.4	17.6
+3	18.4	18.4	21.2	20.0	18.6	19.3
+6	16.2	19.8	21.6	18.8	16.0	18.5
+9	17.6	20.4	24.8	21.6	21.2	21.1
+12	20.2	24.4	25.2*	21.6	22.2	22.7
+15	20.8	22.2	24.0	20.6	22.8	22.1
+18	18.6	21.0	22.8	23.0	23.6	21.8
+21	17.0	18.8	22.5	22.4	19.4	20.0
+24	17.2	19.8	22.4	22.8	22.4	20.9
+27	17.8	20.4	24.0	20.6	23.2	21.2
+31	21.0	21.0	21.6	22.8	20.8	21.4
+34	19.8	22.2	23.2	23.6	25.4	22.8
+36	22.0	24.6*	24.8	24.8	26.8*	24.6
+39	23.6	21.0	24.8	24.6	22.4	23.3
+42	25.8*	24.0	22.8	25.8	23.0	24.3
+45	21.6	21.8	21.6	24.8	20.8	22.1
+52	23.8	23.2	21.6	29.2*	21.4	23.8
+59	15.0	22.4	16.6	24.6	19.6	19.6
+65	16.6	20.0	17.8	23.4	15.2	18.6
+72	17.4	19.0	16.6	24.0	14.0	18.2
+80	17.8	19.2	15.2	----	17.8	16.7

All observations have been expressed relation to Day 0; the first day of patent disease.

*Peak IgG level observed for each calf.

$\bar{x}$ - mean.

TABLE VIII

SERUM IgM LEVELS (mg./ml.) DURING ANAPLASMOSIS IN GROUP II CALVES

Day	Calf Number 164	165	166	167	168	169	$\bar{x}$
-38	0.63	1.50	0.91	----	----	----	1.01
-35	0.68	1.41	0.99	0.93	----	0.97	0.99
-33	0.78	1.51	0.79	----	0.80	0.88	1.06
-31	0.84	1.86	0.85	1.03	0.92	0.88	1.00
-29	0.78	1.83	0.77	0.85	----	----	1.05
-27	0.78	1.55	0.72	0.93	0.76	0.93	0.95
-25	0.90	----	0.72	----	0.73	0.72	0.76
-23	0.82	1.41	----	1.11	0.75	----	1.02
-21	1.38	1.15	0.63	1.02	----	0.82	1.00
-19	----	1.35	0.61	----	0.65	0.79	0.85
-17	1.22	1.17	----	0.98	0.67	0.90	0.98
-14	1.00	1.07	0.70	0.85	----	0.71	0.86
-12	----	----	0.88	----	0.74	0.73	0.78
-11	1.03	1.11	----	0.90	0.75	----	0.94
-8	0.80	0.99	0.67	0.76	0.84	----	0.81
-6	----	----	0.57	0.92	0.69	0.74	0.73
-5	0.73	0.88	----	0.87	0.71	----	0.73
-2	1.23	0.86	0.82	0.78	----	0.74	0.88
0	----	----	1.60	----	0.86	0.74	1.06
+1	1.68	1.12	----	1.53	1.30	----	1.40
+4	1.80	1.55	1.91	2.43	----	1.83	1.90
+6	----	----	2.93	2.26	1.43	1.56	2.04
+7	1.36	2.01	----	d	----	----	----
+8	d	2.23	4.10	----	----	1.95	2.36
+9	----	d	d	----	2.56	d	----
+10	----	----	----	----	d	----	----

All times have been expressed in relation to Day 0; the first day of patent disease.

$\bar{x}$ - mean.

d - died.

TABLE IX

SERUM IgG LEVELS (mg./ml.) DURING ANAPLASMOSIS IN GROUP II CALVES

Day	Calf Number 164	165	166	167	168	169	$\bar{x}$
-39	17.8	18.4	31.0	----	----	----	22.4
-36	18.8	18.8	34.6	----	----	----	24.1
-33	20.6	21.0	25.6	----	----	15.6	20.7
-31	21.6	19.2	30.2	----	18.6	16.0	21.1
-29	19.6	18.2	27.0	----	19.4	----	21.1
-26	19.8	18.3	28.0	21.8	17.6	16.1	20.3
-24	18.3	----	28.0	----	19.4	15.4	20.3
-23	19.0	19.1	----	20.0	18.0	----	19.0
-21	17.3	18.5	25.2	18.6	----	14.3	18.8
-19	----	16.4	25.2	----	19.4	16.6	19.4
-17	16.1	15.8	----	19.2	19.2	12.6	16.6
-14	17.6	15.2	25.6	15.1	----	15.1	17.7
-11	18.9	17.4	----	16.6	21.7	----	18.6
-8	15.8	15.8	28.6	16.8	19.2	19.4	19.3
-6	----	----	27.0	20.4	17.6	13.3	19.6
-5	20.5	17.0	----	17.0	19.8	----	18.6
-2	21.6	19.0	23.2	19.4	----	16.1	19.9
0	----	----	27.8	----	20.2	10.0	19.3
+1	20.6	18.1	----	20.8	19.5	----	19.7
+4	22.0	19.9	29.6	19.2	----	17.7	21.7
+6	----	----	27.8	17.0	21.6	13.4	19.9
+7	16.5	17.8	----	d	----	----	----
+8	d	18.9	28.6	----	----	13.4	----
+9	----	d	d	----	22.0	d	----
+10	----	----	----	----	d	----	----

All times have been expressed in relation to Day 0; the first day of patent disease.

$\bar{x}$ - mean.

d - died.

CHAPTER V

DISCUSSION

Pretreatment Immunoglobulin Levels and the Effects of Splenectomy

The levels of immunoglobulins found in these calves were similar to the values in man although IgG levels were higher (19.8 mg./ml., compared to 12 mg./ml. in man) (Fahey and McKelvey, 1965; Claman and Merrill, 1964). IgG levels varied considerably from one animal to another. (Table III).

Splenectomy depressed IgM levels in Group II (Table II), but IgG values were unaffected. (Table III). Dimopoullos, Foote and Schrader (1959) found that splenectomy in calves induced a transient decline in gamma globulins, of one month duration. Since the calves in the present study were infected 27 days after splenectomy, it is possible that IgG levels could have returned to normal by this time. The prolonged depression of IgM is intriguing but unexplained.

The Kinetics of the Immunoglobulin Response in Intact Calves

The classification of Anaplasma marginale is a subject of considerable controversy, and one of more than academic interest. Theiler (1909) believed it to be a protozoon, although others have since attempted to classify it with the rickettsiae or the viruses. It is feasible that it may not belong to any of these groups of microorganisms.

It is always tempting to draw analogies, and it appears to be a common characteristic of the scientific mind to attempt to classify information, to draw inferences and to detect similarities where possibly none exist. Thus, although the taxonomy of Anaplasma marginale remains sub judice, the disease it produces seems to possess certain clinicopathological features in common with other anemias of an infectious nature, which are caused by hematozoan organisms. These include trypanosomiasis, malaria, piroplasmosis and others. All of these infections are arthropod-borne and manifested by varying degrees of parasitemia and anemia. In many of them the spleen plays a central role in the natural immune status of the host, and also in the development and maintenance of acquired immunity. This is especially so in piroplasmosis (Garnham, 1963). In these diseases, convalescence is often accompanied by persisting latent infection, the relative immune state being what Sergent (1963) has termed premunition.

To this list of similarities we may now add another, namely the occurrence of pronounced macroglobulinemia during the course of the immune response. This has been described in Trypanosoma equiperdum infection in the rabbit (Mattern, Duret and Pautrizel, 1963), T. gambiense in man (Masseyeff and Lamy, 1966), human malaria (Abele et al., 1964) and bovine anaplasmosis (Murphy, Osebold and Aalund, 1966b).

The present study has substantiated the findings of Murphy, Osebold and Aalund (1966b) on a quantitative basis. Substantial increases in IgM levels were found in calves infected with Anaplasma marginale. This macroglobulinemia lasted for at least 90 days in some animals. The reasons for this type of immunoglobulin response remain to be determined. LoSpalluto et al. (1962) have pointed out that antibodies that remain

macroglobulin in nature are mostly formed in response to particulate antigens with a relatively high carbohydrate content, such as typhoid O-antigens and RBC isoantigens. Persistence of antigens probably plays a significant part in determining the length of the IgM response, as suggested by the findings of Uhr and Finkelstein (1963). These authors studied the heterogeneity of the immunoglobulin response to bacteriophage ØX 174 in the guinea pig. They felt that the short-lived nature of the IgM response seen after a single injection of phage was caused by antigen depletion.

The function of IgM antibodies is still uncertain. Talmage, Freter and Taliaferro (1956) and others have demonstrated that IgM hemolysins have greater hemolytic efficiency than IgG, on a mole per mole basis. Recently, Hill and Robbins (1966) reported that the mouse-protecting activity of IgM anti-pneumococcal antibodies was some 100,000 times greater than that of IgG antibodies. They concluded that this difference was not solely due to the larger molecular weight of IgM antibodies. Several investigators have shown that the threshold dose of antigen for IgM synthesis is lower than that for IgG (Svehag and Mandel, 1962; Uhr and Finkelstein, 1963). These observations tend to suggest that macroglobulins may act as an 'acute phase reactant system', which is very sensitive to low levels of antigen (e.g. in the early stages of a systemic infection), and very efficient as an interim body defense mechanism, until the synthesis of IgG antibodies has been stimulated.

An explanation such as this is no doubt teleological to a degree, and it certainly does not explain why the majority of antigens elicit only a transient IgM response, whereas others produce only IgM antibodies, regardless of the stage of immunization (LoSpalluto et al.,

1962). The immunoglobulin response to Anaplasma marginale appears to fall between these two extremes, with an apparent preponderance of macroglobulin antibodies. One should not overlook the fact that neither in this study nor in that of Murphy, Osebold and Aalund (1966a) was there any evidence to suggest that the IgM-IgG sequence detected was actually related to specific 'protective' antibodies that may occur in anaplasmosis. Virtually nothing is known of the immunological mechanisms that are involved in curtailing the growth of anaplasma, and hence in determining the outcome of the infection. However, there is also no reason to suppose that any unique processes are involved, as distinct from any other microbial infection.

Whatever the antibodies in anaplasmosis may be, it appears that they are predominantly macroglobulin in nature, especially in the early patent period. This is a time of crisis in the disease. During a period of 7-10 days after the onset of patent parasitemia, the rate of multiplication of the organism determines severity of the anemic crisis, and in part, the likelihood of recovery, together with other factors such as the alacrity of the erythropoietic response.

It is evident from the experiments of Jones et al. (1967) and from the present study, that antigenic stimuli must occur in infected animals prior to detectable parasitemia. Both the CF antibody and the IgM response preceded Day 0 by several days. (Figures 12,A and 14). It is not known if Anaplasma marginale undergoes an exoerythrocytic stage of development, such as occurs in infections with Plasmodium spp. The presence of this early immunological response suggests that this is feasible. However, it may also be indicative of the initial invasion of the RBC by the organism, in a form which is not recognizable by conven-

tional light microscopy.

Effects of Splenectomy on the Kinetics of the Immunoglobulin Response

An intriguing observation in this study was the fall in IgM and IgG levels during the prepatent period of the disease in the splenectomized calves. The significance of this is uncertain. Dimopoullos, Schrader and Foote (1960) described a fall in alpha, beta and gammglobulins during the same period in their splenectomized animals. This was regarded as a nonspecific "debilitating effect" on serum proteins. The apparent absence of the phenomenon in intact calves (Figure 12) awaits explanation.

The present investigation has shown that the IgM response was significantly delayed in splenectomized animals. This agrees with the electrophoretic data of Dimopoullos, Schrader and Foote (1960). They found that alpha and beta-globulin levels in splenectomized calves increased around Day 0, which is very similar to the time of the initial increase in IgM levels observed in Group II. (Figure 12,A).

Murphy, Osebold and Aalund (1966a) failed to detect any differences in the kinetics of the immunoglobulin response in intact and splenectomized animals, but as has been mentioned previously, there appeared to be a delay in the appearance of CF antibodies in their splenectomized cattle. This appears to be characteristic of the disease pattern in asplenic animals, as has been shown by Norman (1966) and confirmed in the present study.

It is a salient fact that although the onset of the IgM response was delayed in Group II, these calves still formed appreciable amounts of this immunoglobulin before death, and the mean rate of IgM synthesis

appeared to be very similar to that of Group I. (Figure 12,A). Since all of Group II died, direct comparison of the time and magnitude of maximal IgM levels is impossible. However, since the time of onset was delayed, and the rate of synthesis similar to that of Group I, one may infer that the peak of the response was also delayed.

When the values of peak parasitemia in Group I were compared with the time of peak IgM levels, relative to the day of peak parasitemia, a linear relationship was apparent. (Appendix Figure 15). The correlation between these two variables ($r=0.96$) was significant at the 5% level. A similar relationship was found between the time of peak CF antibody titers relative to the time of peak parasitemia, and the magnitude of peak parasitemia ($r=0.94$). This would be expected since the day of maximal CF antibody titers correlated well with the day of maximal IgM levels ($r=0.97$). Furthermore, in Group I there was a linear relationship between the number of days taken to reach peak IgM levels and the intensity of the parasitemia ($r=0.92$). (Appendix Figure 16). This was relationship just below the 5% level of significance.

These observations suggested that those calves that reached maximal IgM levels earlier developed lower levels of parasitemia. Admittedly, both the range of parasitemia (12-19%) and the number of animals (5) on which these statistics are based are small, but the question would certainly merit further investigation.

These findings, together with the fact that the immunoglobulin response in anaplasmosis appear to be predominantly IgM in nature, have led the author to propose the following hypothesis on the role of the spleen in immunogenesis to this infection.

It appears from our data that the intact calf can detect lower

levels of anaplasma antigens, than its splenectomized counterpart. This would explain the earlier immunological responses in the former group. Assuming that the immunoglobulin response encompasses the production of specific 'protective' antibodies, this delay could be a significant factor in the pathogenesis of the higher parasitemias observed in asplenic calves. Thus it is postulated that the IgM response in these animals is 'out of phase' with the development of parasitemia.

A hypothesis such as this must have its obvious defects, especially since it is based on rather meager data. One of these is the assumption that the relationship between the intensity of parasitemia and the time of peak IgM levels would apply to splenectomized animals. It is possible that it might not. If one regards the intensity of parasitemia as a measure of the antigen load on the animal's reticuloendothelial system (RES), then this is obviously much greater in splenectomized calves, than in the controls. This might result in an accelerated and enhanced immunoglobulin response, which could mask an actual impairment of reactivity. Uhr and Finkelstein (1963) found that the IgM response in guinea pigs was maximal once a certain threshold dose of antigen was exceeded. In this study, the correlation between maximal IgM levels and maximal parasitemia was poor. However, direct comparison between the antibody response to an inert antigen, and that to a viable microorganism, is difficult especially since the "dose" of antigen in the latter case is largely out of the control of the investigator. It is not known if the rate of proliferation of the microbial agent would influence the responsiveness of the immunoglobulin-producing mechanisms, after the minimal antigen threshold had been reached.

The above hypothesis probably only offers a partial explanation of

the effects of splenectomy on the severity of anaplasmosis, since it disregards the phagocytic functions of the spleen. This organ contains only one-seventh of the total RES, but its phagocytic propensities appear to be greater than this figure would suggest (Ellis and Smith, 1966). There is evidence that splenic macrophages are more efficient in phagocytosis than the Kupffer cells of the liver, especially when titers of opsonizing antibodies are low (Benacerraf, Sebestyen and Schlossman, 1959). This would again implicate the spleen in the early stages of a systemic infection, when there is rapid multiplication of the micro-organism, in the absence of specific immunity.

In summary it has been postulated that the effects of splenectomy on the severity of Anaplasma marginale infection in cattle can be explained in the following terms: (1) decreased sensitivity to low levels of parasite antigens, resulting in delayed macroglobulin antibody formation and hence greater parasitemia (the function of these antibodies in the immune state remains speculative). (2) impaired phagocytic clearance of the parasite due to removal of a small, but significant, part of the RES.

These two factors are obviously closely inter-related. Phagocytosis of the antigen is believed to be an essential step in the formation of antibodies, and the production of specific opsonons would in turn aid phagocytosis. Although it is convenient to consider 'cellular' and 'humoral' factors in immunity as separate entities, it is clear that the immune state to any antigen is the result of a complex interplay of many mechanisms which do not operate in isolation.

Summary and Conclusions

Two groups of four-month-old calves (Group I intact, Group II splenectomized) were infected with Anaplasma marginale, and the kinetics of the serum immunoglobulin response was studied throughout the ensuing disease.

In Group I the concentration of IgM started to increase 7 days before the onset of patent disease and rapidly reached a peak of 200% above the preinfection mean value for this group. This macroglobulinemia persisted for over 90 days in three of the calves.

All the calves in Group II died. The onset of the IgM response in this group was delayed until Day 0, the first day of patent disease.

The concentration of IgG in Group I increased 11 days after the initial increase in IgM levels and slowly rose to a peak around Day +40. The increase in IgG levels was not large, compared to that of IgM, nor was the response as prolonged.

It appeared that those calves in Group I, which reached maximal IgM levels earlier, showed a less intense parasitemia. It was therefore postulated that the effects of splenectomy on the severity of anaplasmosis are explicable on the basis of: (1) decreased sensitivity to low levels of parasite antigens in splenectomized animals, causing a delay in antibody production and, (2) impaired phagocytic clearance of the parasite due to the absence of the spleen.

This study has opened a number of avenues for further research. The above hypothesis on spleen function in anaplasmosis requires further experimental investigation, especially since it is not known if the relationship between the time of maximal IgM titers and the intensity of

parasitemia would apply to splenectomized animals. The significance of the pronounced IgM response requires elucidation. It seems probable that these immunoglobulins are involved in the phagocytic clearance of anaplasma. An investigation of the relative opsonizing properties of IgG and IgM fractions from the sera of infected animals is therefore desirable. The significance of the phagocytic properties of the spleen likewise needs investigation. This could be achieved by a study of the RES clearance efficiency of intact and splenectomized animals, both before and after infection with Anaplasma marginale and by in vitro experiments on the isolated spleen.

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APPENDIX

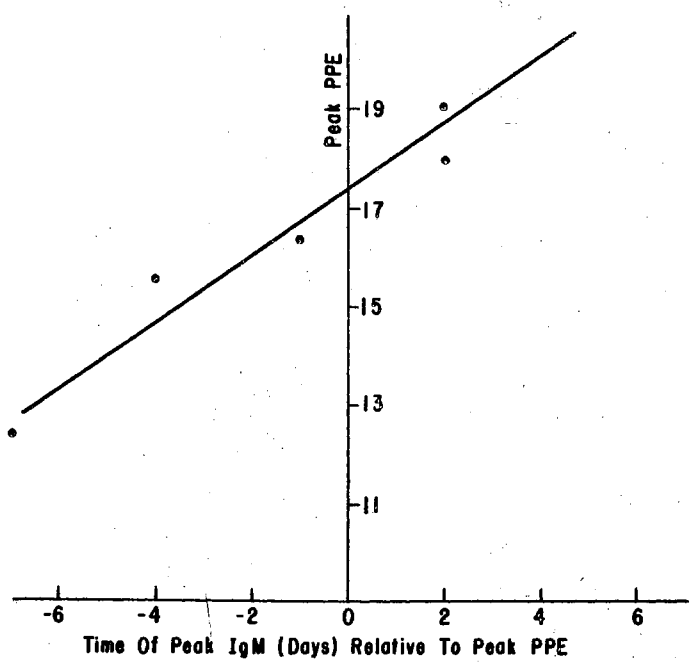

Figure 15. Relationship between the time of maximal IgM response relative to the time of peak parasitemia, and the magnitude of peak parasitemia, in Group I calves.

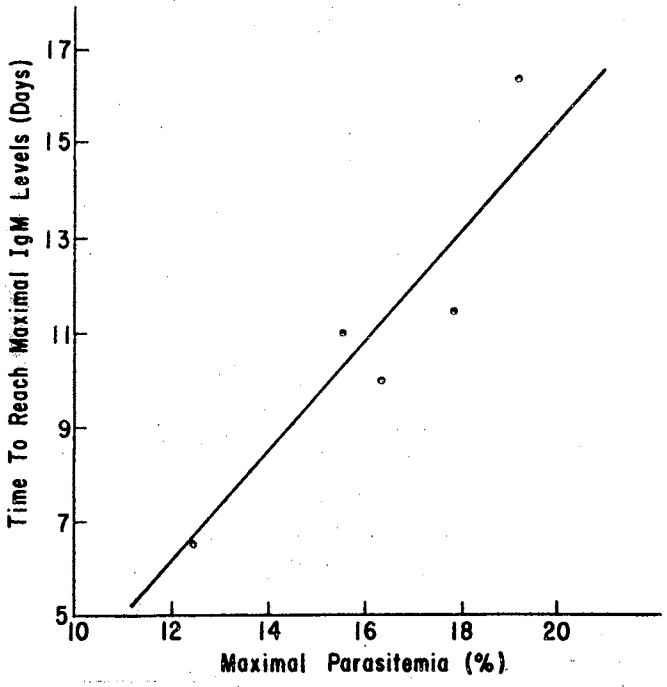

Figure 16. Relationship between the number of days to reach maximal IgM levels, and the magnitude of peak parasitemia, in Group I calves.

VITA

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