COMPARISON OF IMMUNOGLOBULINS OF NORMAL ANIMALS AND ANIMALS INFECTED AND IMMUNIZED WITH TRYPANOSOMA BRUCEI.

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INTRODUCTION

A. GENERAL FEATURES OF IMMUNOGLOBULINS AND THE IMMUNE RESPONSE

i) Definition of immunoglobulin:- Immunoglobulins are defined as proteins of animal origin endowed with known antibody activity, and certain proteins related to them by chemical structure and hence antigenic specificity (WHO, 1964). The chemical structure of these proteins has been studied in detail and comprehensive reviews have been written on the subject (Cohen and Porter, 1964; Fudenberg, 1965; Cohen and Milstrein, 1967).

ii) Classes of immunoglobulins: It is now known that antibodies are globulins which occur in the gamma- or beta- fractions of this serum protein. These globulin fractions are referred to as immunoglobulins because they take part in immune reactions. Before antibodies became known as immunoglobulins, they were classified on the basis of their function, e.g. lysins, agglutinins, precipitins. However, with the increase in the knowledge of the nature, physical properties and molecular structure of the immunoglobulins that constitute antibodies, 5 main classes have been found in humans, and these have been designated IgG, IgM, IgA, IgD and IgE. Each of these have subclasses and WHO (1964) recommends a system of nomenclature for human immunoglobulins and the application of the same principles to the nomenclature of immunoglobulins in animals. Neoh et al. (1973) examined the plasma of 90 species of animals for homologues of the 5 classes of human immunoglobulins by cross-reactivity tests and found that they occurred in almost all primates. Similar classes have been found in several mammalian species.

iii) Methods of characterising immunoglobulins: In order to study and characterise immunoglobulins, it is necessary to obtain them in a reasonable degree of purity.

For this purpose, various methods are used to separate the different immunoglobulins from other serum components.

Salt fractionation is one of the oldest methods to be used for separating the serum proteins and is based on differences in the solubility of particular proteins in high concentrations of sodium sulphate, ammonium sulphate or zinc sulphate. These salts cause the precipitation of gamma-globulins which must then be subjected to chromatography, gel filtration or electrophoresis to obtain the different fractions for characterisation. A good description of salt fractionation is given by Heide and Schwick (1973).

Electrophoresis is also used for separating serum proteins. This may be carried out in a supporting medium such as polyacrylamide gel (zone electrophoresis) or in an entirely liquid phase (free boundary electrophoresis). An extension of this method of separating proteins is referred to as immunoelectrophoresis and consists of first separating the proteins by zone electrophoresis using a supporting medium such as agar and then allowing antiserum to diffuse in to the agar from a trough cut parallel to the line of protein migration. For a complete description of immunoelectrophoretic techniques, Ouchterlony and Nilsson (1973) may be referred to.

Immunoglobulins may be characterised by ultracentrifugation which depends on the differential sedimentation of molecules of different sizes and shapes when subjected to high speed centrifugation. The technique is described in detail by Stanworth (1973).

Gel filtration (column chromatography) and ion-exchange chromatography are now in common use for the separation and characterisation of immunoglobulins. Gel filtration, also known as exclusion chromatography, separates proteins on the basis of molecular size.

It is carried out in columns packed with a proprietary dextran, e.g. Sephadex (Pharmacia Fine Chemicals, London) which is available in various grades with different exclusion limits. Sephadex G200 is commonly used for the separation of immunoglobulins. The distribution of the molecules in the eluate can be determined by quantitative ultraviolet absorption measurements and specific characterisation of the proteins can be done by immuno electrophoresis (Fahey and Terry, 1973).

Immunodiffusion is another method of analysing and quantitating immunoglobulins. It involves a precipitation reaction between soluble proteins which diffuse through a gelled medium such as agar. The double diffusion method in which antibody and antigen diffuse from different wells in the medium can be used to give a semi-quantitative estimate of the amount of antibody present in serum samples. The single radial diffusion method involves the mixing of one of the reactants with the agar and allowing the other to diffuse into the agar. Precipitation occurs where there is optimal concentration of antigen and antibody producing a ring around the well. The diameter of the ring is proportional to the concentration of antigen or antibody which can be accurately determined by comparison with a known standard (See Ouchterlony and Nilsson, 1973).

iv) Methods of enhancing the immune response: The immune response may be enhanced by immunization, the purpose being to increase the resistance of the animal to the disease against which it has been immunized and this forms the basic aim of practical immunology. Increased immunoglobulin levels may result from:-

a) the administration of killed or inactivated organisms or their products. Disease organisms or their products may be inactivated by treatment with chemicals such as formalin or beta-propiolactone or the organisms may be physically

disrupted by sonication. Alternate freezing and thawing is sometimes employed to kill organisms. Microbial products such as toxins may also be inactivated by chemical treatment which alters them in such a way that their disease-producing power is removed while their immunogenicity is retained.

b) the use of live, attenuated organisms. Attenuation may be achieved by passage in laboratory animals, embryonated eggs or tissue culture. Such live vaccines produce more persistent and higher levels of immunoglobulins than inactivated vaccines probably because the organisms multiply in the body thereby increasing the amount of antigen and providing continuous stimulation.

c) Infection and treatment. In many cases it may not be possible either to grow the organism to produce enough antigen or to attenuate it for use as a vaccine. In such circumstances, animals may be immunized either by simultaneously administering the infectious agent with a therapeutic agent or allowing the infection to establish before instituting treatment.

B. PURPOSES OF STUDY OF IMMUNOGLOBULINS IN TRYPANOSOMIASIS

The study of immunoglobulins in trypanosomiasis has as its ultimate aim the discovery of a method of producing active protection against the disease (Desowitz, 1960[b]). Since it is realised that to achieve this goal, a thorough understanding of the disease and its causal agent is necessary, the immunological studies have been concerned with all aspects of the disease. In general, the purposes of the studies may be summarised as follows:-

1. To find a reliable means of diagnosis: In many infections it is not possible to demonstrate trypanosomes directly due to scanty parasitaemia and the organism involved may not be infective to laboratory animals or does not grow in artificial culture media. A variety of serological tests dependent on antibodies have been used for the diagnosis of trypanosomiasis in the past, e.g. complement fixation, agglutination, lysis and immune adherence. More recently, considerable attention has been given to the diagnostic value of raised immunoglobulin levels in serum and cerebrospinal fluid, which Mattern et al. (1961) found to be associated with T. gambiense infection e.g. Lumsden (1966), Binz et al. (1968), and Luckins (1972, 1973, 1974).

2. To examine the possibility of immunization: All authorities agree that complete control of tsetse-transmitted trypanosomiasis can only be achieved by the eradication of the vector. However, the huge cost and the practical problems involved necessitate the exploration of other methods of control. Since control by chemotherapy and chemoprophylaxis have the inherent danger of inducing the development of drug-resistant strains of trypanosomes, the possibility of immunization is being examined (Gray, 1967). This involves logically the identification of trypanosomal antigens which stimulate protective immunity, the identification of protective immunoglobulins and the development of methods of stimulating an increase in their production. Soltys (1964, 1965) used killed trypanosomes inactivated by various chemicals while Cunningham and Grainge (1965) and Wilson and Harley (1968) used infection and drug-treatment to produce immunity.

3. To study the immune response in relation to antigenic variation It is known that trypanosomiasis is characterised by a relapsing parasitaemia with peaks of parasitaemia followed by crises which

coincide with the appearance of antibodies in the serum (Gray 1967, 1970). Variations of the antigenicity of the trypanosome are associated with the relapsing parasitaemia and antibody formation. Although it has been established that these antibodies, which are variant-specific, can influence the course of antigenic variation (Gray, 1962), the exact relationship is not clear. Since it is the variable antigens of the trypanosome that stimulate the production of protective antibody (Gray, 1970), an understanding of the nature of the relationship is a pre-requisite for progress in research on immunity to trypanosomiasis.

4. To elucidate the pathology of trypanosomiasis: It is now recognised that a range of immunological abnormalities occur in trypanosomiasis (Murray et al, 1974) and attempts are in progress to find an immunological basis for some of the pathological features seen in the disease. Boreham (1968) found increased kinin activity in the sera of rabbits and cattle with chronic trypanosomiasis and postulated that the liberation of kinins was due to antigen-antibody reaction. Subsequently, Boreham and Kimber (1970) demonstrated immune complexes on the endothelium of renal blood vessels. Goodwin and Guy (1973) associated the pathogenesis of T. brucei infection in rabbits with allergic reactions and believed that one of the causes of death was renal failure.

5. To understand the epidemiology and epizootiology of trypanosomiasis: The need to provide a basis for serological typing of trypanosome strains was pointed out by Gray (1966). It may be possible to determine the number of antigenically different strains of a common trypanosome in a specific locality by serological methods and then to study certain aspects of the epidemiology and epizootiology of trypanosomiasis by serological surveys (Gray, 1967).

C. METHODS FOR MEASURING SERUM PROTEIN CHANGES IN NORMAL, INFECTED AND IMMUNIZED ANIMALS WITH SPECIAL REFERENCE TO TRYPANOSOMIASIS

Serum protein changes occur in many diseases, e.g. malaria, kala azar, leprosy and trypanosomiasis (Woodruff, 1959; McKelvey and Fahey, 1965; Watson and Chirieleison, 1966). Such changes may be revealed by a study of the different serum protein components of normal animals and animals infected or immunized against a particular disease. The following methods can be used to study these differences:-

1) Chemical assay of the serum proteins: The total protein content of the serum may be determined by the biuret test or the Kjeldahl method while the zinc sulphate precipitation test can be used to measure the amount of gamma-globulin present. However, in order to determine the amount of particular serum protein fractions, the method of electrophoretic separation must also be utilised. These tests have been employed to study the immune response and measure the serum protein changes that occur in infected or immunized animals as compared with normal animals. For example, Desowitz (1959) studied the immune response of resistant and susceptible breeds of cattle to trypanosomal challenge by chemical and electrophoretic analyses of sera in conjunction with other tests, and found that while no marked alteration occurred in the alpha - or beta-globulins, challenge was always followed by an increase in the gamma-globulin content, which is usually preceded by an initial fall. Extending his studies to antelopes, Desowitz (1960a) observed a marked increase in the beta - and gamma-globulins after challenge and noted that in some cases, albumin decreased as the infection progressed. Bideau et al. (1966) carried out immunoelectrophoretic studies of serum from cattle infected with trypanosomes and concluded that immunoglobulin changes were comparable to those seen in human patients.

Cornille and Hormung (1968) used paper electrophoresis to examine sera from T. rhodesiense - infected patients and carried out quantitative studies of the IgM levels by immunodiffusion using serial dilutions of test sera and a fixed dilution of antiglobulin.

2) Serological tests: These tests are used primarily to detect and quantitate antibodies and may be used for the same purposes with regard to samples of pure immunoglobulins of different classes. The serological tests that have been used to detect changes in antibody content of the serum of infected or immunized animals have been discussed in reviews by Weitz (1958, 1970), Gray (1967), Killick-Kendrick (1968) and Lumsden (1973). These include the traditional complement fixation test, precipitation, neutralization and agglutination tests, and the modern fluorescent antibody technique.

Serological tests have been widely applied to the study of the antibody response in several diseases including trypanosomiasis. The precipitin test was used by Gray (1966) to demonstrate precipitins in the sera of Zebu and Ndama cattle after infection with T. vivax and T. congolense. Soltys (1957) used the agglutination test to detect the presence and quantitate agglutinating antibodies in the sera of rabbits infected with brucei-group trypanosomes and T. congolense. The specificity of the direct fluorescent antibody test was shown by Weitz (1963) while Latif and Adam (1973) reported on the specificity of the indirect test. Bailey et al. (1967) and Wilson et al. (1967) used the indirect method to demonstrate the presence of antibodies in infected cattle.

Serological methods are also frequently used to measure the amounts of immunoglobulins of different classes i.e. IgG and IgM, in sera from normal, infected and immunized animals. Immunodiffusion techniques are most commonly adopted nowadays for this purpose.

The double diffusion method in agar gel has been used as a semi-quantitative method for estimating immunoglobulins by Mattern (1968) and Cornille and Hornung (1968). For more accurate determinations of immunoglobulin, single radial diffusion techniques are usually employed e.g. McKelvey and Fahey (1965), Binz et al. (1968) and Luckins (1972, 1973, 1974).

D. CHARACTERISTICS OF THE ANTIBODY RESPONSE IN TRYPANOSOMIASIS

It has long been known that trypanosomiasis is characterised by an increase in the level of gamma-globulins (Woodruff, 1959; Smithers and Terry, 1959). Even before serum globulins were classified into alpha-, beta-, and gamma-fractions, it was known that in trypanosomiasis and some other diseases, serum proteins then referred to as globulin and euglobulin increased and produced a positive serum-formalin test which could be used for diagnostic purposes (Desowitz, 1970). Since these observations, serum protein changes in trypanosomiasis have been investigated more fully, particularly in human infections e.g. Mattern et al. (1961), Mattern et al. (1964), and Lumsden (1966). Changes occurring in domestic and wild animals have also been examined recently. Many of the investigations into the immune response in trypanosomiasis were carried out with a view to using this as a screening test in the diagnosis of the disease.

A constant finding in all the investigations is a high and persistent rise in the IgM level of infected humans and animals. For example, Mattern (1961) found a beta-macroglobulinaemia in sleeping sickness patients in West Africa and this finding has since been confirmed by Lumsden (1966) in East Africa and Binz et al. (1968) in Zaire. Similar elevated IgM levels have been found in animal trypanosomiasis (Seed et al., 1969; Van Meirvenne et al., 1972; Luckins, 1972,

1973, 1974; Clarkson and Penhale, 1973). Seed and his colleagues (1969) found that in rabbits infected with T. gambiense, IgM levels are raised early in the infection, the level reaching a peak at about 18 days after infection and remaining high for up to 117 days. Lumsden (1966) also found that in Rhodesian Sleeping Sickness, the rise in IgM levels occurred early in the infection "within at most a few weeks" while in his studies of bovine trypanosomiasis, Luckins (1972) found that high IgM levels occurred within 2 weeks of infection. This early increase in IgM levels was also noted by other workers (Van Meirvenne et al., 1972; Schindler, 1973). IgG levels on the other hand, increased later in the infection and the increase is less marked. Clarkson and Penhale (1973) found no increase in the level of this immunoglobulin until about 40 days after infection while Luckins (1973) and Luckins (1974) found only a "slight increase" and "little change" respectively within a few weeks after infection. With regard to IgA levels in the serum of infected animals, Van Meirvenne et al. (1972) observed a temporary decrease in dogs infected with T. rhodesiense while normal levels were maintained in dogs infected with T. gambiense. Clarkson and Penhale (1973) found no change in IgA levels in cattle infected with different strains of T.vivax; IgA levels also remained unchanged in monkeys (Macaca mulatta) infected with different strains of T. rhodesiense, T. gambiense and T. brucei (Houba et al., 1969).

The nature of the elevated IgM and IgG levels has been investigated by several workers. Houba et al. (1969) found that even after absorption with homologous antigen, "there was no significant change in the IgG and IgM values" and they concluded that the increased values are due to a large extent to non-specific antibodies. Mackenzie et al. (1972) found that in rabbits, non-trypanosome

specific antibodies contribute to the elevated IgM levels in trypanosomiasis while Freeman et al. (1970) concluded that the non-specific component of IgG antibodies in trypanosomiasis of the rhesus monkey may be as much as 95%. More recently, Mackenzie and Boreham (1974) found increased amounts of naturally occurring tissue autoantibodies in rabbits infected with T. brucei and claimed that these contribute partly to the raised IgM levels in trypanosomiasis. They also suggest that depression of certain T-Cell functions may release antibody secreting B-Cell descendants from T-Cell control resulting in elevated IgM levels.

The specific antibodies found in trypanosome infections are produced in response to a variety of trypanosomal antigens. Some of these antigens are common to different species and strains and are referred to as "common antigens" while others are associated with the surface coat of the organism and are known as "exoantigen" (Weitz, 1960). Investigations have shown that only the antibodies produced against the exoantigens are protective and that the protective activity is highly type-specific. Attempts are now being made to characterise pure immunoglobulin fractions containing these antibodies on a functional basis. Seed et al. (1969) found agglutinating activity initially in the IgM fraction, but later in the infection, this activity was found in both the IgG and IgM fractions. Strong agglutinating activity was also found in the IgM fraction by Van Meirvenne et al. (1972), but both groups of workers found precipitating activity in the IgG fraction only. In rabbits infected with T. brucei and T. equiperdum, Seed (1972) found that early in the infection, protective activity occurred in the IgM fraction, later occurring in the IgG fraction (21 days after infection) with the IgM fraction losing its protective activity.

The appearance of neutralizing antibodies to T. brucei in rats on the 6th day of infection (Cunningham et al., 1963) and these antibodies reaching a peak between 20-30 days in cattle infected with T. congolense (Wilson and Cunningham, 1972) would suggest that neutralizing activity occurs in the IgM fraction. This is supported by the finding by Luckins (1974) that the increase in the level of IgM, which occurred within 2 weeks of infection with T. congolense in cattle, coincided with the appearance of neutralizing antibodies.

Although Seed et al.,(1969) believed that the persistently high levels of IgM were due to antigenic variation, with each variant stimulating the production of variant-specific antibody, it is now known that much of the increased IgM is due to non-trypanosome specific antibodies (Houba et al., 1969; Freeman et al., 1970; Mackenzie et al., 1972; Mackenzie and Boreham, 1974). The observation that the appearance of trypanosome-specific antibodies coincides with the decline of parasitaemia andthe appearance of a new variant suggests that these antibodies may be responsible for antigenic variation and the relapsing parasitaemia that is characteristic of trypanosomiasis.

E. OBJECTIVE AND OUTLINE OF CURRENT WORK

The objective of the current work was to compare the immunoglobulins of normal animals with those of animals infected and immunized with T. brucei. It was thus necessary to obtain a suitable trypanosome strain and experimental host, to practice and become proficient in techniques to characterise immunoglobulins produced in trypanosome infections in terms of quantity and quality (e.g. class and function) and to design experiments to produce the material required for these purposes.

The strain of T.brucei used was identified as TREU 1168 and was derived from T. brucei TREU 730, ETat 5. The latter had a short laboratory history of rodent passage and caused reliably heavy infections in mice. It also produced chronic infections in rabbits. These animals were therefore chosen as experimental hosts for the work.

The methods used to measure the amounts of antibody - containing serum protein fractions were electrophoresis, the zinc sulphate test for gamma-globulin, immunodiffusion in agar for IgG and IgM estimation and an agglutination test for functional activity. The experimental animals were infected by inoculation with the stabilate of T. brucei and the method adopted to immunize animals was infection and drug-treatment.

In order to become proficient in the techniques to be used, preliminary work was carried out on material obtained from normal rabbits and mice and on sera donated by members of staff of the protozoology section. Following this, two experiments were performed to produce material for more detailed comparative study.

MATERIALS AND METHODS

ANIMALS: Rabbits and mice were used as experimental animals. Adult Porton Cross rabbits supplied by Edinburgh University Centre for Laboratory Animals, and weighing between 2 to 4 kg were used in the experiments. These were kept in 24 x 12 x 18 ins. aluminium cages (Forth-Tech Services Ltd, Dalkeith, Midlothian) and maintained on standard pelleted SG1 small animal diet (Oxoid Ltd, Southwark Bridge Rd., London). The rabbits were used for the production of sera from infected and immunized animals.

The mice used were Theiler's Original Strain obtained from A. Tuck and Sons, Laboratory Animal Breeding Station, Rayleigh, Essex. The adult mice weighing approximately 20 g each, were housed in 13 x 6 x 5 ins. plastic mouse cages (Type NKP - M2 (North Kent Plastics, Dartford). They were maintained on pelleted McGregor's Laboratory Animal diet (Quay side Mills, Leith, Scotland). The mice were used for the production of immune sera and for challenge experiments.

TRYPANOSOME: A stabilate prepared from Trypanosoma brucei, TREU 730, supplied from the trypanosome bank of the C.T.V.M., was prepared and preserved according to the method of Polge and Soltys (1957) as modified by Cunningham et al. (1963). The procedure was as follows: the content of a capillary tube containing T. brucei TREU 730 was drawn into a 1 ml syringe. Approximately 0.5 ml of Locke's solution (see appendix) was drawn into the syringe and mixed by inverting the syringe several times. Three mice were each inoculated intraperitoneally with 0.15 ml of the trypanosome suspension and examined daily for parasitaemia. On day 2, when examination of tail blood showed adequate parasitaemia (more than 50 organisms per microscope field; x 40 objective) the mice were bled by cardiac puncture after ether anaesthesia using heparin as anticoagulant at the rate of 5 I.U./ml of mouse blood.

A prefreeze trypanosome count on the pooled blood from the 3 mice gave an average of 99.4 organisms per microscope field. A total of 1.8 ml of blood was obtained to which 0.15 ml of sterile glycerol was added to give a final concentration of 7.5% of glycerol v/v. After mixing well, the test tube containing the blood was placed in an ice bath while the blood was dispensed into capillary tubes with a sterile pasteur pipette. After sealing both ends of the three-quarter filled capillaries with a microburner, they were placed in a rubber-stoppered test tube containing chilled methanol. The test tube was then placed in an insulating jacket which was in turn placed in a solid CO_2 cabinet (- 79° C) for gradual cooling overnight. The following day, the stabilate was transferred to the permanent trypanosome bank and coded as TREU 1168. An indication of the survival rate obtained by performing a trypanosome count on the day-old frozen stabilate gave an average of 94.6 organisms per microscope field. Capillary tubes of the stabilate were taken out as required during the course of the experiments.

METHODS FOR ENUMERATING TRYPANOSOMES: Trypanosomes were counted by the following 2 methods.

(i) Examination of wet blood films - The number of trypanosomes seen in a microscope field (x 40 objective) were counted using a tally counter. At least 10 microscope fields were counted and the average taken.

(ii) Examination by haemocytometer - The cell of the assembled Neubauer haemocytometer was filled with the well-mixed 1/100 dilution of the trypanosome suspension, by means of a pasteur pipette. After allowing about 5 minutes for the trypanosomes to settle, the organisms in the 4 corner squares (i.e. 0.4 mm^3) were counted under the x 40 objective and the

number per ml calculated from this figure.

SERUM COLLECTION: Blood from the rabbits was obtained from the marginal ear vein while that from mice was obtained by cardiac puncture after ether anaesthesia. Clotting was hastened by incubating the blood at 37°C and the serum transferred into test tubes after 'ringing' the clot. The sera were then centrifuged at 4°C at 1500 r.p.m. for 20 minutes to remove blood cells that may be present. The serum samples were stored in labelled bottles at -15°C.

SERUM CHEMISTRY: Chemical assays for total proteins and gamma-globulins were performed. The total protein was estimated by the biuret test (Henry et al., 1957), using 'Versatol' (William R. Warner, Eastleigh, Hants) as the protein standard. The tests were carried out as follows:

Reagent blank - 5 ml 3% Na OH

Standard - Rinsed 0.1 ml Versatol into 4.9 ml 3% Na OH

Serum Samples - Rinsed 0.1 ml of serum into 4.9 ml 3% Na OH

1 ml of biuret reagent was added to each tube and mixed. Tests were read after at least 15 minutes using an SP 200 spectrophotometer (Pye Unicam Ltd., Cambridge, England) at 545 nm. The total protein contents of the sera were obtained by substituting the following formula:-

$$\text{Protein, g/100 ml} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times \text{g. protein in standard.}$$

The gamma-globulin contents of the sera were estimated by the zinc sulphate test. The tests were carried out as described in the Ministry of Agriculture, Fisheries and Food (MAFF) manual of Veterinary Investigation Laboratory Techniques (Page 128) as follows:- 0.1 ml of each serum sample was pipetted into 2 test tubes. 5.9 ml of distilled water was taken into one of the tubes and mixed.

To the second tube 0.9 ml of distilled water was added, mixed and then 5.0 ml of zinc sulphate was added. After mixing well by inversion, both tubes were left for 1 hour before reading the test using an EEL Colorimeter. For each sample, the tube containing serum and distilled water only was used to zero the instrument. The gamma-globulin concentration in g./100 ml serum was read from a table in the MAFF manual.

The relative proportions of the different serum protein fractions were determined using the Millipore Clinical Electrophoresis System (Millipore (U.K.) Ltd., Millipore House, Abbey Rd., London NW10). The procedure involved separating the protein fractions by electrophoresis on 3" x 1" cellulose acetate strips bonded to mylar (Millipore U.K. Ltd.), using barbital buffer pH 8.6, for 23 minutes. The strips were then stained with Ponceau-S stain, dehydrated in ethanol and cleared in a mixture of ethyl acetate and glacial acetic acid (30:70). Readings and chart recordings (see Figure 1) were made with the 'Phoroscope' Densitometer (Millipore (U.K.) Ltd.).

The absolute concentration of the different protein fractions was obtained by calculation using the relative percentage figures and the total protein content from the biuret test.

IMMUNOLOGICAL DETERMINATIONS:

A. AGGLUTININ TITRATION: The development and persistence of agglutinins in the experimental animals and the presence of agglutinins in pure immunoglobulin fractions of selected sera were determined using the agglutination test described by Cunningham and Vickerman (1962).

The procedure followed was similar to that described by Lumsden et al. (1973) except that serial doubling dilutions of the sera were used in the present work.

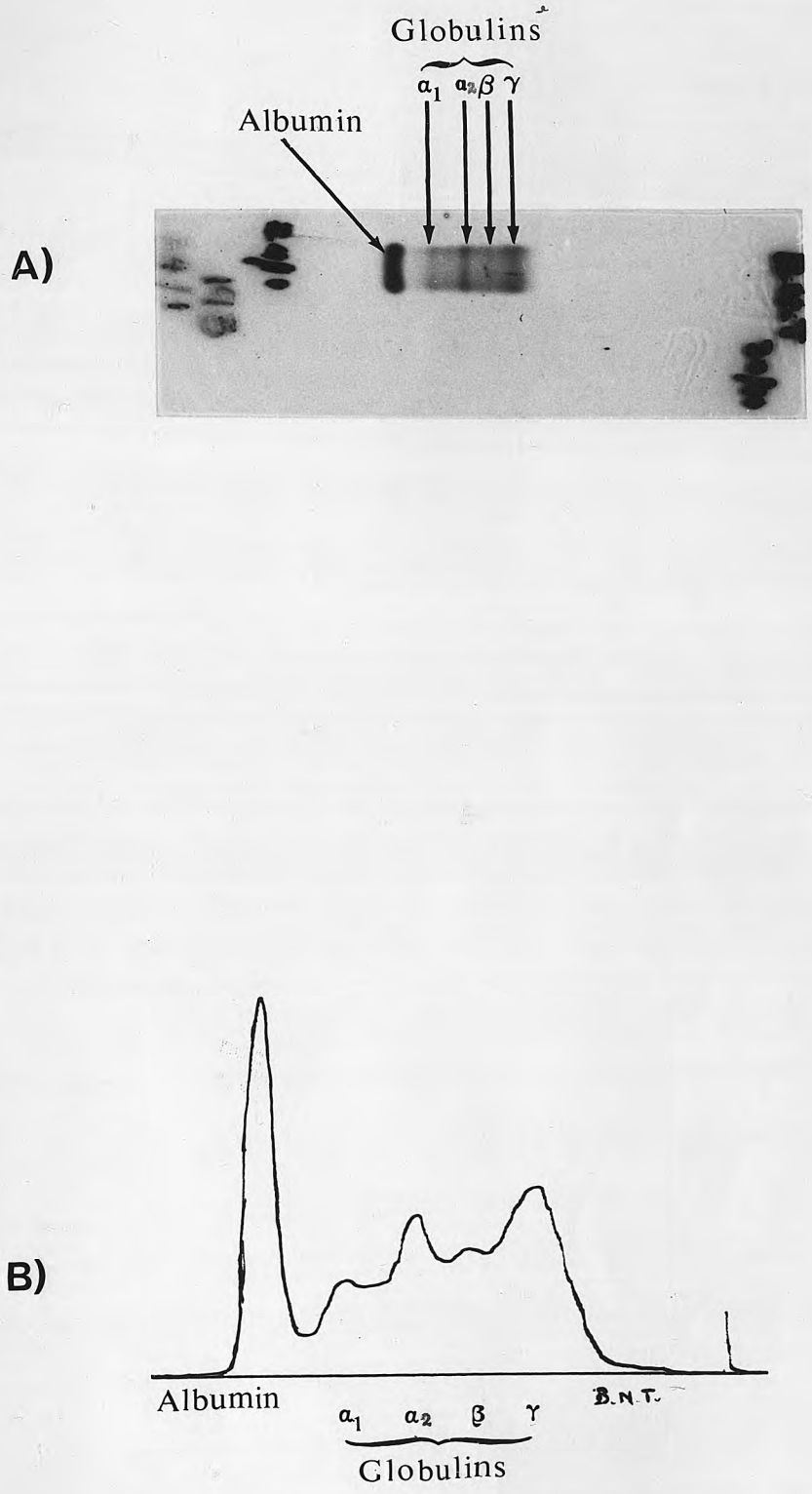


Fig. 1. Electrophoretic pattern of serum from Rabbit 193, obtained 14 days after infection with T. brucei TREU 426.

A) The stained separated serum protein fractions on 'Phoroslide' and

B) The corresponding peaks on a chart recording

The test was carried out as follows:-

1. Serial two-fold dilutions of each serum sample from 1/10 to 1/40960 were prepared in perspex agglutination trays using the buffer solution ABB-8.0 (see appendix) as the diluent.
2. The dilutions of sera were placed as drops on siliconized slides in a humidity chamber together with a drop of buffer and a drop of 1/10 dilution of the preinfection or pretreatment serum sample as controls.
3. Small amounts of an appropriate stabilate antigen were added to each of the dilutions of serum by means of a micropipette.
4. The slides were examined under the x 10 objective of the microscope, after 30 minutes incubation at room temperature.

The method of scoring was similar to that descfibed by Lumsden et al. (1973) but the titre of the serum was taken as the greatest dilution showing 1 + agglutination.

B. IMMUNOGLOBULIN ESTIMATION: IgG and IgM concentrations in serum samples were determined by double diffusion and single radial diffusion techniques. The double diffusion technique was carried out according to the method described by Mattern (1968). The test was carried out using ionagar 1% (Nordic Pharmaceuticals, Fraburg Ltd., Berks) in 0.85% sodium chloride (w/v) adding 0.01% sodium azide to prevent bacterial and fungal growth. Approximately 20 ml of melted agar, cooled to 56°C, was poured on a clean 10 x 10 cm glass plate on a flat level surface.

After the setting of the gel, the plates were placed in a refrigerator for at least 1 hour to facilitate the cutting out of the wells. Groups of 8 peripheral and one central wells were punched out using a special gel cutter. For IgG estimations, a specific goat anti-rabbit IgG serum (Miles Labs Ltd., Bucks) was placed in the central well and two-fold dilutions of the sera under test were placed serially in the peripheral wells. In the preliminary experiments, test sera were diluted from 1/1 to 1/128 but in later experiments, samples were diluted from 1/32 to 1/4096. For IgM estimations, a specific goat anti-rabbit IgM sera (from the same commercial source) was placed in the central well and dilutions of the test sera from 1/1 to 1/128 were placed serially in the peripheral wells (see plates 1-4).

Single radial diffusion tests were carried out as described by Fahey and McKelvey (1965). The plates were prepared by melting 3% agarpse (Miles Laboratories Ltd., Bucks) in a boiling water bath, cooling to 56°C and them mixing with an equal amount of preheated (56°C) specific goat anti-rabbit IgG or IgM sera (Nordic Pharmaceuticals Ltd., Berks) previously diluted 1/10 with 0.3M phosphate buffer pH 8.0. This gave a final concentration of 1.5% agarose, and after mixing well, the mixture was poured on 10 x 10 cm glass plates in 20 ml amounts. The gel was allowed to set in the same way as the double diffusion plates. When ready, 36 wells arranged in 6 rows of 6 wells each were punched out using a special plate holder and template.

The serum samples from each rabbit were applied to a row of wells starting with the pooled preinfection sample in the 1st well on the left and the 5th week sample in the last well on the right as shown in Plate 5. The diameter of the precipitin rings were measured with a x 8 ocular magnifying micrometer (Flubacher, Zurich), after 24 and

48 hours incubation in a humid chamber at 4°C for IgG and IgM respectively.

SERUM FRACTIONATION: Selected serum samples were fractionated by gel filtration on a 1.5 x 85 cm column of Sephadex G200 (Pharmacia) using 0.1M Tris-HCl buffer, pH 8.0, (see appendix). Material from the first two ascending and descending peaks corresponding to IgG and IgM were collected. Each of these fractions was concentrated to its original volume by dialysis against polyethylene glycol 6000 (British Drug Houses Ltd., Poole, England). The purity of the fractions was then ascertained by immunoelectrophoresis using 1% ionagar (Nordic Pharmaceuticals) in barbitone acetate buffer, pH 8.6. The 10.5 x 8 cm agar plates for electrophoresis were prepared in the same manner as the immunodiffusion plates. When the plates were ready, a set of 3 parallel troughs and 4 wells were cut through the agar using a special template (see Plate 6) The agar from the wells was then lifted out and IgG and IgM fractions applied to the top 2 and bottom 2 wells respectively. The plate was then placed in the electrophoresis bath and separation allowed to take place at a constant voltage of 150 volts for 90 minutes. At the end of this period, the plate was taken out, the agar from the troughs lifted and the bottom of each trough sealed with melted agar. Specific goat anti-rabbit IgG was applied to the top trough, polyvalent goat anti-rabbit serum to the middle trough and specific goat anti-rabbit IgM in the bottom trough. The plates were incubated for 24 hours at 4°C before examination.

MATERIAL FOR STUDY

Material for examination was obtained in the course of the following studies:-

Preliminary Experiments: Studies with normal rabbit sera and the sera of rabbits infected with trypanosomes.

The object of these preliminary experiments was to gain experience of the techniques to be used in the subsequent experiments and to gather base-line data for future comparative work. The following materials were examined:-

1) Normal rabbit sera - 10 serum samples from 6 normal rabbits prior to use in Experiment1) were examined for

i) Total protein content

ii) Proportions of protein fractions by electrophoresis and absolute concentration of components by calculation.

iii) Gamma-globulin content

iv) IgG and IgM levels

v) Immuno-electrophoretic precipitin pattern.

2) Sera collected at varying intervals from Rabbit 193, from the 4th to the 25th day after infection with T.brucei TREU 426. These sera were kindly provided by Dr. A. G. Luckins. Examinations were carried out for

i) Total protein content

ii) Proportions and absolute concentrations of protein fractions

iii) IgG and IgM levels

iv) Agglutinin titres against T. brucei TREU 426

v) Immuno-electrophoretic precipitin pattern

3) Sera from 2 rabbits infected with T. gambiense, strains M8 and M10 transmitted by tsetse flies, obtained before infection and at varying intervals during the course of the infections. The sera were kindly provided by Dr. A. R. Gray and were examined for

i) Total protein content

ii) Proportions and absolute concentrations of the protein fractions

iii) IgG and IgM levels.

Experiment 1: Comparative study of immunoglobulins of normal, infected and immunized rabbit sera.

The aim of this experiment was to compare the development and persistence of immunoglobulins in infected and immunized animals. Six rabbits were used and these were divided into 3 groups of 2 rabbits each. Rabbits 1 and 2 (Group 1) were infected with T. brucei stabilate TREU 1168 and the infection allowed to run an untreated course. Rabbits 3 and 4 (Group 2) were immunized by infection with parasitaemic blood from Rabbit 1 and treated with suramin B.P. at the rate of 20 mg/kg body weight (Soltys, 1957) administered intravenously as a 10% solution using the marginal ear vein. Rabbits 5 and 6 (Group 3) were not infected but were treated with suramin at 20 mg/kg to provide sera from drug-treated animals for control purposes.

Sera were collected from the 6 rabbits before infection or drug-treatment and afterwards at weekly intervals for 5 weeks and the following examinations were carried out:-

i) Total protein content

ii) Proportions and absolute concentrations of different protein fractions.

iii) Gamma-globulin content

iv) IgG and IgM levels by double diffusion and single radial diffusion techniques

v) Agglutinin titres against T. brucei TREU 1168

In addition, selected serum samples were fractionated and agglutination tests performed with the pure IgG and IgM fractions.

Experiment 2: Comparative study of immunoglobulins in normal and immunized mice.

This experiment was carried out to extend the comparison of the

immunoglobulins in normal and immunized rabbits to a second host, i.e. mice, and to provide an opportunity for direct tests of the effect of immunization by infection and drug-treatment since the number of rabbits available in Experiment 1 was inadequate for the latter purpose.

Seventy five mice were used. Five were bled for normal sera and the remaining 70 were divided into 2 groups of 35 mice each, designated Group A and Group B.

The mice in Group A were infected with T. brucei TREU 1168 and examined daily for parasitaemia. The inoculum was prepared by taking the contents of 8 capillaries of the stabilate in 8 ml of Locke's solution and each mouse received 0.2 ml of the trypanosome suspension intraperitoneally. On day 3, when the mice had a high parasitaemia, they were treated with diminazene aceturate B.vet.C (Berenil, Hoechst Chemicals Ltd.), at the rate of 25 mg/kg (Herbert and Lumsden, 1968; Lumsden et al., 1973) administered subcutaneously. The mice in Group B, which had not been infected, were treated in the same way with the drug and kept for control purposes.

Groups of 5 mice from Group A and Group B were bled for serum each week for 5 weeks. The sera of each subgroup were pooled and stored in labelled bottles at -15°C. All the sera were examined for:-

i) Total protein content

ii) Proportions and absolute concentrations of the different protein fractions

iii) Gamma-globulin content

iv) Agglutinin titres against T.brucei TREU 1168

Agglutinin titres of pure IgG and IgM fractions of selected sera were also determined.

Challenge Experiments: The remaining 10 mice in each group were subdivided into 2 groups each of 5 designated A1, A2, B1 and B2 and challenged with 2 doses of T. brucei TREU 1168 as follows:

The contents of 5 capillaries of the stabilate were taken into 2.5 ml of chilled phosphate buffer ABP 8.0 (see appendix). After mixing well, 0.1 ml of the trypanosome suspension was transferred to 9.9 ml of phosphate buffer in a universal bottle to give a 10^{-2} dilution. A trypanosome count on this dilution showed that it contained 95×10^3 trypanosomes per ml of the suspension. One subgroup from each of the two main groups was inoculated intraperitoneally with 0.2 ml of the original trypanosome suspension while the other 2 subgroups were each inoculated with 0.2 ml of the 10^{-2} dilution of the suspension as shown below:

GROUP	SUBGROUP	INOCULUM DILUTION	APPROX. NO. OF TRYPS.
A	A1	undiluted	19×10^5
	A2	10^{-2}	19×10^3
B	B1	undiluted	19×10^5
	B2	10^{-2}	19×10^3

All the mice were observed for parasitaemia and deaths for 31 days.

RESULTS

Preliminary experiments: Studies with normal rabbit sera and the sera of rabbits infected with trypanosomes.

1) Observations on normal rabbit sera.

The total protein content, relative proportions and absolute concentrations of the different serum protein fractions of the 6 normal rabbits are shown in Table 1. The absolute concentrations of the gamma-globulin fraction obtained by calculation and zinc sulphate tests, are compared in Table 2. These results show that the proportion of gamma-globulin in normal rabbit sera was not more than 10% of the total serum proteins and the absolute concentrations of this protein fraction did not exceed 1 g /100 ml of serum.

The IgG and IgM levels in the sera are shown in Table 2. The IgG titre was high in normal serum (>1/128) while the IgM titre was very low.

The immunoelectrophoretic precipitin patterns obtained with the normal sera from the 6 rabbits were similar and the pattern obtained with a sample from Rabbit1is shown in Plate 6A. This plate also shows that the commercially prepared anti-sera to rabbit IgG and IgM were specific.

2) Observations on sera from Rabbit 193.

Table 3 shows the total protein content, and the relative and absolute concentrations of the serum protein fractions of sera from Rabbit 193 during infection with T. brucei TREU 426. The results show an increase in the total protein content from 5.68 g/100 ml 4 days after infection to a maximum of 8.49 g/100 ml 18 days after infection, after which the level started falling. During the course of the infection, there was a marked fall in the proportion of albumin from 48% 4 days after infection to 33% 3 weeks later, while the

Table 1. Showing the total protein content, relative proportion and absolute concentrations of the different serum protein fractions of the sera from normal rabbits.

SAMPLE		TOTAL PROTEIN	ALBUMIN		ALPHA-GLOBULIN		BETA-GLOBULIN		GAMMA-GLOBULIN	
		g/100 ml	Proportion %	conc'n g/100 ml	Proportion %	conc'n g/100 ml	Proportion %	conc'n g/100 ml	Proportion %	conc'n g/100 ml
R1	1	5·28	71	3·69	7	0·36	15	0·79	7	0·36
	2	5·55	69	3·82	6	0·33	14	0·77	11	0·61
R2	1	4·92	56	2·75	16	0·78	11	0·54	17	0·83
	2	5·56	63	3·5	23	1·27	7	0·38	9	0·50
R3	1	5·94	68	4·03	20	1·18	7	0·41	5	0·29
	2	5·04	52	2·62	27	1·36	11	0·55	10	0·50
R4	1	5·87	69	4·05	8	0·46	15	0·88	8	0·46
	2	5·93	57	3·38	25	1·48	10	0·59	8	0·47
R5	1	6·2	64	3·96	8	0·49	23	1·42	5	0·31
R6	1	4·6	66	3·03	5	0·23	23	1·05	6	0·27

Table 2. Showing the gamma-globulin content and the IgG and IgM titres of sera from Rabbits 1-6 before infection or drug-treatment

RABBIT	GAMMA-GLOBULIN g/100 ml		IgG TITRE	IgM TITRE
	Calculation	$ZnSo_4$ test		
1	0·48*	0·49*	> 1/128	1/2
2	0·66*	0·53*	"	1/2
3	0·39*	0·41*	"	1/1
4	0·46*	0·53*	"	1/4
5	0·31	0·58	"	1/2
6	0·27	0·45	"	1/4

* Average of 2 estimations

Table 3. Showing the total protein content, relative proportion and absolute concentrations of the different serum protein fractions of sera collected from Rabbit 193 during infection with *T.brucei* TREU 426.

DAYS AFTER INFECTION	TOTAL PROTEIN	ALBUMIN		ALPHA-GLOBULIN		BETA-GLOBULIN		GAMMA-GLOBULIN	
	g/100 ml	Proportion %	conc'n g/100 ml	Proportion %	conc'n g/100 ml	Proportion %	conc'n g/100 ml	Proportion %	conc'n g/100 ml
4	5·68	48	2·72	19	1·07	18	1·02	15	0·85
7	5·87	51	2·99	17	0·99	18	1·05	14	0·82
10	5·68	43	2·44	17	0·96	16	0·93	24	1·40
12	6·03	41	2·47	16	0·96	20	1·20	23	1·38
14	5·62	35	1·96	23	1·29	16	0·89	26	1·46
18	8·49	33	2·80	13	1·10	14	1·18	40	3·39
25	7·50	36	2·70	14	1·05	14	1·05	36	2·70

proportion of gamma-globulins increased within the same period from 15% to 40% and the absolute concentration from 0.85 g/100 ml to 3.39 g/100 ml. The alpha- and beta- globulins showed no significant change.

The gamma-globulin content determined by the zinc sulphate method and the IgG and IgM titres of the sera from Rabbit 193 are shown in Table 4. The end point of the IgG titration was not reached because the sera were diluted to 1/128 only but the IgM titre increased 16 times from 1/4 4 days after infection to 1/64 by 18 days after infection. By 25 days after infection the titre had declined to 1/16.

The results of the agglutination tests using sera from Rabbit 193 and stabilate TREU 426 as antigen are also shown in Table 4. All the serum samples collected up to 18 days after infection had good agglutinating activity at dilutions of 1/20480, but the strongest agglutination was obtained from the 12th day sample which gave a 2+ agglutination at 1/20480. The agglutinin titre fell to 1/2560 by the 25th day after infection.

3) Observations on serum from Rabbits infected with T. gambiense strains M8 and M10: Table 5 shows the total protein content, IgG and IgM titres of sera from the 2 rabbits infected with T. gambiense strains M8 and M10. The relative proportions of the serum protein fractions could not be determined due to poor electrophoretic separation. In both rabbits, the total protein content and the IgM titres showed a rise similar to that which occurred in T. brucei - infected Rabbit 193, but the end point of the IgG titration was again not reached.

Table 4. Showing the gamma-globulin content, IgG, IgM and agglutinin titre of sera from Rabbit 193 during infection with T.brucei TREU 426.

DAYS AFTER INFECTION	GAMMA-GLOBULIN g/100 ml	IgG TITRE	IgM TITRE	AGGLUTININ TITRE AGAINST TREU 426
4	0·85	> 1/128	1/4	1/20480
7	0·82	"	1/8	1/28480
10	1·40	"	1/8	1/28480
12	1·38	"	1/16	1/20480
14	1·46	"	1/32	1/20480
18	3·39	"	1/64	1/20480
25	2·70	"	1/16	1/2560

Table 5. Showing the total protein content, IgG and IgM titres of sera from 2 rabbits infected with different strains (M8 and M10) of T.gambiense.

Rabbit	Serum Sample	Days after infection	Total Protein g/100 ml	IgG Titre	IgM Titre
M8	P 5	0	7·7	> 1/128	1/2
	P 6	16	7·36	"	1/4
	P 7	30	7·78	"	1/16
	P 8	48	8·64	"	1/8
M10	P 9	0	5·76	"	1/4
	P 10	14	7·06	"	1/8
	P 11	29	6·9	"	1/32
	P 12	49	6·63	"	1/16

Experiment 1: Comparative Study of immunoglobulins of normal, infected and immunized rabbits.

The results of the determinations for total protein content and the relative and absolute concentrations of the different serum protein fractions of the sera from the 6 experimental rabbits are shown in Tables 6-8. The results show that in the 2 rabbits in Group 1, which were infected and left untreated, the total protein content increased from a preinfection level of 5.2 g/100 ml to 11.1 g/100 ml in Rabbit 1 and from 5.2 g/100 ml to 9.4 g/100 ml in Rabbit 2. In Rabbit 1, the relative proportion of albumin fell from 63% to 34% 5 weeks after infection (Table 6), while the proportion of gamma-globulin increased from 9% to 34% (Table 6 and Figure 2). The electrophoretic patterns of sera from Rabbit 1 are shown in Figure 3, which clearly shows the increase in the proportion of gamma-globulins. Although the absolute concentration of the gamma-globulins showed a corresponding increase, the fall in the proportion of albumin was not reflected in the absolute concentration of this protein during the course of the infection. The response of Rabbit 2 which died in the fourth week after infection was similar to that of Rabbit 1 in that while the proportion of albumin decreased during the infection, the gamma-globulin showed a corresponding increase relative to the other protein as well as in absolute amount. In both rabbits, the alpha- and beta-globulins did not show any marked change.

Rabbits 3 and 4 of Group 2, which were treated with suramin 1 week after inoculation of parasitaemic blood, did not show any change either in the total protein content or the relative proportion of the serum protein fractions. The total protein content and the concentrations of the different serum protein fractions of sera from

Table 6. The total protein content and the relative and absolute concentrations of the different serum protein fractions of sera from Rabbits 1 and 2 infected with T.brucei TREU 1168.

RABBIT NO.	SERUM SAMPLE	TOTAL PROTEIN g/100 ml	ALBUMIN		ALPHA-GLOBULIN		BETA-GLOBULIN		GAMMA-GLOBULIN	
			Proportion %	conc'n g/100 ml	Proportion %	conc'n g/100 ml	Proportion %	conc'n g/100 ml	Proportion %	conc'n g/100 ml
	Preinfection	5·2	63	3·27	7	0·36	21	1·09	9	0·46
	Week 1	9·4	60	5·64	8	0·75	22	2·06	10	0·94
	Week 2	7·5	47	3·52	11	0·83	28	2·10	14	0·75
1	Week 3	6·2	39	2·41	12	0·74	23	1·43	26	1·61
	Week 4	8·5	38	3·23	10	0·85	20	1·70	32	2·72
	Week 5	11·1	34	3·77	12	1·33	20	2·22	34	3·77
	Preinfection	5·2	60	3·12	6	0·31	23	1·19	11	0·57
	Week 1	5·8	58	3·19	8	0·46	25	1·45	9	0·50
	Week 2	7·2	51	3·67	8	0·57	27	1·94	16	1·15
2	Week 3	7·9	38	3·00	11	0·31	21	1·65	30	2·37
	Week 4	9·4	36	3·38	12	0·47	20	1·88	32	3·01
	Week 5	DEAD	--	--	--	--	--	--	--	--

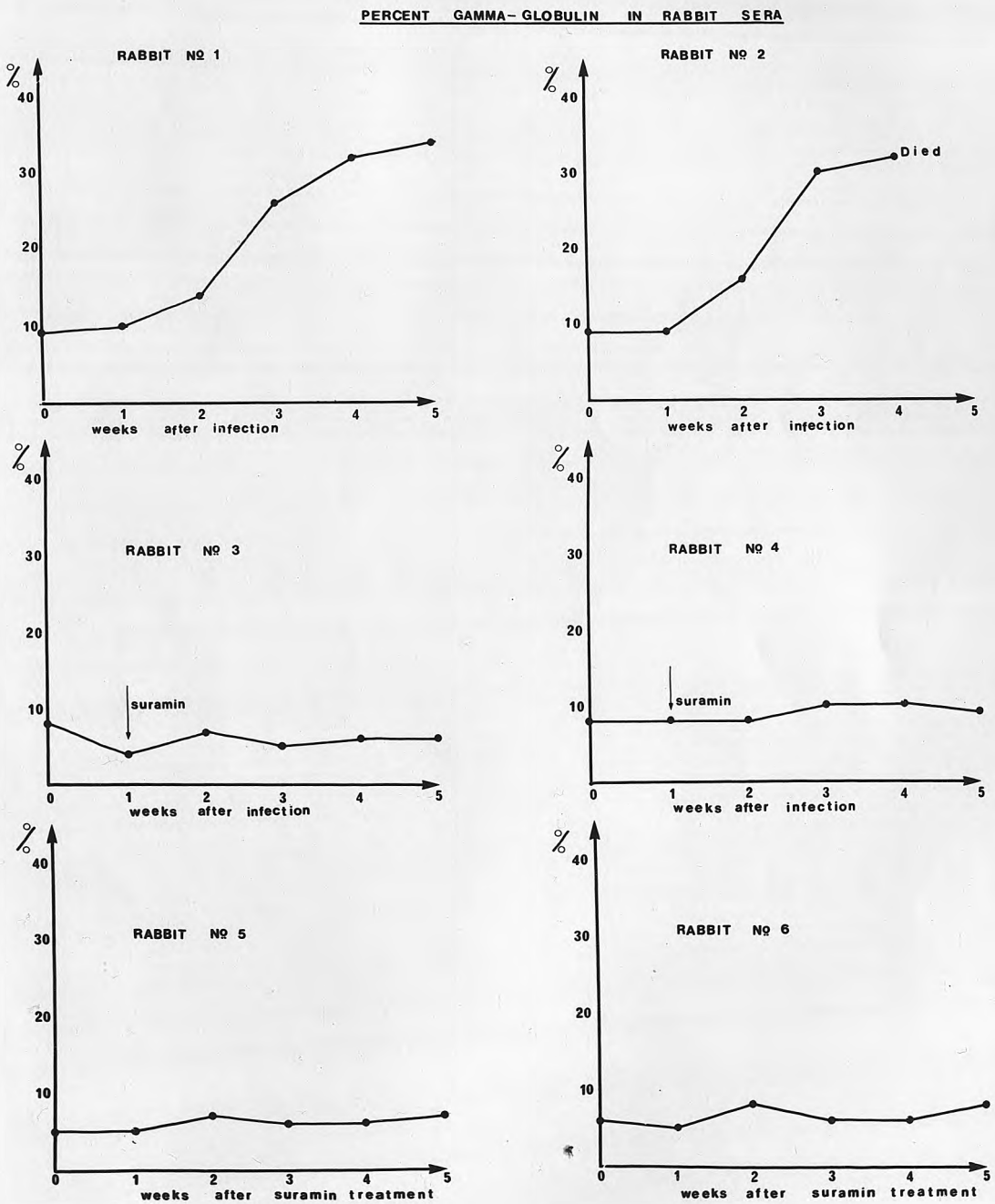
Table 7. The total protein content and the relative and absolute concentrations of different serum protein fractions of sera from Rabbits 3 and 4, which were inoculated with parasitaemic blood and treated with suramin one week later.

RABBIT NO.	SERUM SAMPLE	TOTAL PROTEIN g/100 ml	ALBUMIN		ALPHA-GLOBULIN		BETA-GLOBULIN		GAMMA-GLOBULIN	
			Proportion %	conc'n g/100 ml	Proportion %	conc'n g/100 ml	Proportion %	conc'n g/100 ml	Proportion %	conc'n g/100 ml
3	Preinfection	6·1	60	3·66	6	0·36	24	1·46	10	0·61
	Week 1	6·9	74	5·10	6	0·41	16	1·10	4	0·27
	Week 2	10·7	68	7·27	11	1·76	14	1·49	7	0·74
	Week 3	7·1	72	5·11	9	0·63	16	1·13	5	0·35
	Week 4	8·8	74	6·51	9	0·79	11	0·96	6	0·56
	Week 5	7·9	70	5·53	11	0·86	13	1·02	6	0·47
4	Preinfection	7·6	62	4·71	8	0·61	22	1·67	8	0·60
	Week 1	5·1	72	3·67	4	0·20	16	0·89	8	0·40
	Week 2	5·7	71	4·04	5	0·28	16	0·91	8	0·45
	Week 3	7·2	69	4·96	5	0·36	16	1·15	10	0·72
	Week 4	5·1	66	3·36	5	0·25	19	0·96	10	0·51
	Week 5	6·3	68	4·24	6	0·37	17	1·07	9	0·56

Table 8. The total protein content and the relative and absolute concentrations of different serum protein fractions of sera from Rabbits 5 and 6, which were not infected, but were treated with suramin.

RABBIT NO.	SERUM SAMPLE	TOTAL PROTEIN g/100 ml	ALBUMIN		ALPHA-GLOBULIN		BETA-GLOBULIN		GAMMA-GLOBULIN	
			Proportion %	conc'n g/100 ml	Proportion %	conc'n g/100 ml	Proportion %	conc'n g/100 ml	Proportion %	conc'n g/100 ml
	Pretreatment	6·2	64	3·96	8	0·49	23	1·42	5	0·31
	Week 1	5·7	60	3·42	7	0·39	25	1·42	7	0·39
	Week 2	6·4	66	4·22	6	0·38	22	1·41	6	0·38
5	Week 3	5·8	68	3·94	7	0·41	19	1·10	6	0·34
	Week 4	6·3	61	3·84	8	0·50	24	1·51	7	0·44
	Week 5	5·4	66	3·43	8	0·43	21	1·13	5	0·27
	Pretreatment	4·6	66	3·03	5	0·23	23	1·05	6	0·27
	Week 1	4·8	67	3·21	4	0·19	24	1·15	5	0·24
	Week 2	5·4	63	3·40	6	0·32	23	1·24	8	0·43
6	Week 3	5·8	64	3·71	7	0·41	23	1·33	6	0·34
	Week 4	6·3	62	3·91	6	0·37	26	1·63	6	0·37
	Week 5	5·4	68	3·13	6	0·32	26	1·40	8	0·43

Fig. 2


PERCENT GAMMA-GLOBULIN IN RABBIT SERA
RABBIT Nº 1
RABBIT Nº 2
Died
RABBIT Nº 3
suramin
RABBIT Nº 4
suramin
RABBIT Nº 5
RABBIT Nº 6
%
40
30
20
10
0
1
2
3
4
5
weeks after infection
weeks after suramin treatment

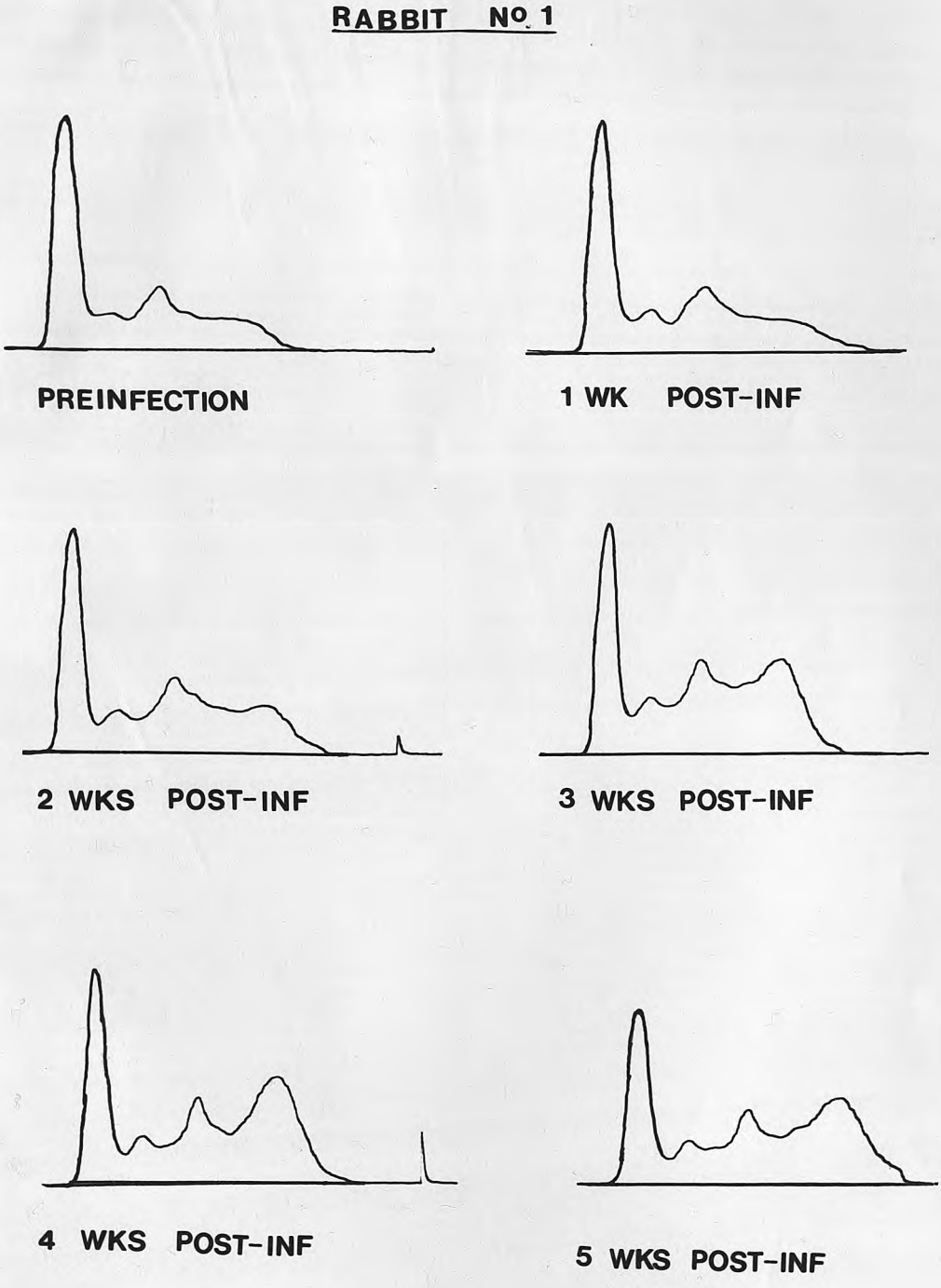


Fig. 3. Electrophoretic patterns of sera from Rabbit No. 1 before and after infection with T. brucei TREU 1168. An increase in the peak corresponding to the gamma-globulin fraction (see fig 1) can be seen.

Rabbits 5 and 6, which acted as the drug-control group, also did not show any significant change.

Table 9 shows the gamma globulin, IgG and IgM content of sera from Rabbits 1 and 2. In these rabbits, the IgG titres of the whole sera increased slightly to twice the preinfection level of 1/512 after an initial fall. Whereas in Rabbit 1, the titre remained at this level until the 4th week after infection, in Rabbit 2, it reached a peak of 1/4096 at week 3 before the level declined (Table 9 and Figure 4). The IgM titres increased from a preinfection level of 1/2 to 1/64 in Rabbit 1 and 1/128 in Rabbit 2, 3 weeks after infection (Table 9 and Figure 5).

The remaining 4 rabbits of Groups 2 and 3 did not exhibit any consistent change in the IgG titre of their sera, as determined by the double diffusion technique. However, with the exception of Rabbit 3 which showed a slight increase in its IgM titre in the 2nd week after inoculation with parasitaemic blood, the IgM levels remained at a uniformly low level (Figure 5). These results were confirmed by immunoglobulin estimations by the single radial diffusion technique. This test did not reveal any significant difference in the level of IgG in the sera of the rabbits but for IgM, it showed a consistent increase in the diameter of the precipitation ring with sera of Rabbits 1 and 2 (Plate 5 and Table 10).

Agglutination tests with whole sera from Rabbits 1 and 2 showed that antibody titres reached a peak of 1/40960, 3 weeks after infection in both animals and then started to decline. The pure IgG fractions of sera obtained from Rabbit 1, one week and 3 weeks after infection did not possess any agglutinin activity but the fraction obtained 5 weeks after infection had an agglutinin titre of 1/20. The agglutinin

Table 9. The gamma-globulin content, IgG, IgM and agglutinin titres of whole sera from Rabbits 1 and 2 infected with *T.brucei*. The agglutinin titres of IgG and IgM fractions of selected sera are also shown.

RABBIT NO.	SERUM SAMPLE	GAMMA-GLOBULIN (g/100 ml)		IgG TITRE	IgM TITRE	AGGLUTININ TITRES		
		Calculation	$ZnSo_4$ test			WHOLE SERUM	IgG fraction	IgM fraction
1	Preinfection	0·46	0·49	1/512	1/2	Negative	Negative	Negative
	Week 1	0·94	0·41	1/256	1/8	1/10240	"	1/640
	Week 2	0·75	0·49	1/1024	1/32	1/20480		
	Week 3	1·61	1·07	1/1024	1/64	1/40960	"	1/80
	Week 4	2·72	0·97	1/1024	1/32	1/20480		
	Week 5	3·77	1·10	1/512	1/160	1/160	1/20	1/40
2	Preinfection	0·57	0·53	1/512	1/2	---		
	Week 1	0·50	0·38	1/256	1/8	1/10240		
	Week 2	1·15	0·58	1/1024	1/32	1/10240	Negative	1/80
	Week 3	2·37	1·55	1/4096	1/128	1/40960	Not done	1/160
	Week 4	3·01	1·60	1/512	1/64	1/20480	1/80	1/40
	Week 5	DEAD	--	--	--	--	--	--

Table 10. IgG and IgM titration of rabbit sera obtained before and after infection or drug-treatment by the single radial diffusion technique.

	IgG						IgM					
	Preinfection	Wk1	Wk2	Wk3	Wk4	Wk5	Preinfection	Wk1	Wk2	Wk3	Wk4	Wk5
				(mm)						(mm)		
R1		5·8	7·1	7·5	7·1	7·1		9·3	9·6	10·3	11·1	11·6
R2		5·8	6·8	6·8	7·5	-		9·1	9·6	10·7	10·7	-
R3	*5·07	5·0	5·2	5·0	5·1	5·2	*8·9	8·5	8·6	8·9	8·9	8·8
R4		5·2	5·3	5·2	5·2	4·8		9·1	9·2	9·2	9·0	9·0
R5		4·6	4·6	4·5	4·5	4·4		8·7	8·9	8·8	9·0	8·7
R6		5·2	5·2	5·1	5·2	5·0		8·9	9·2	8·8	8·8	9·0

* Mean.

Fig. 4

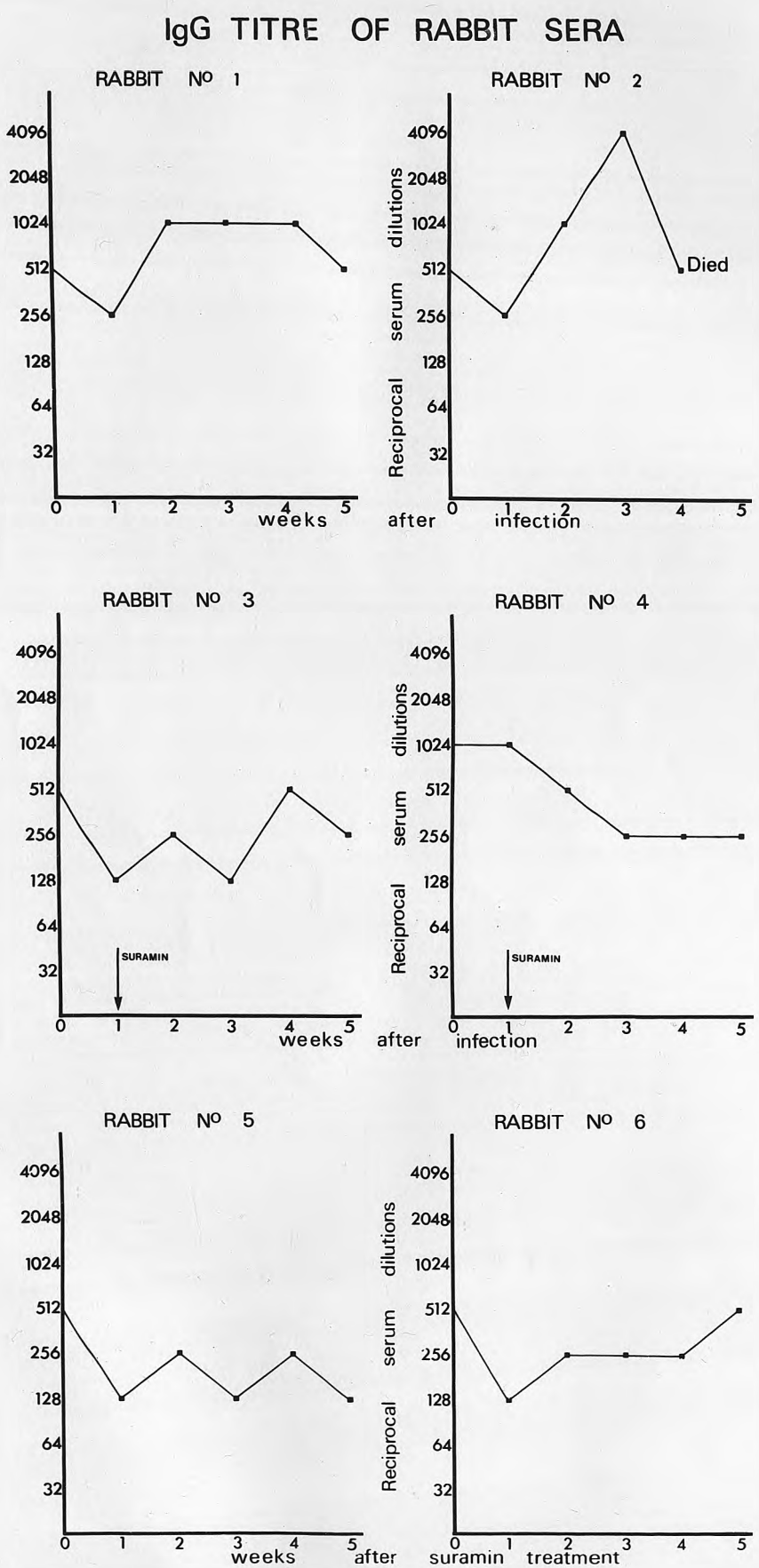
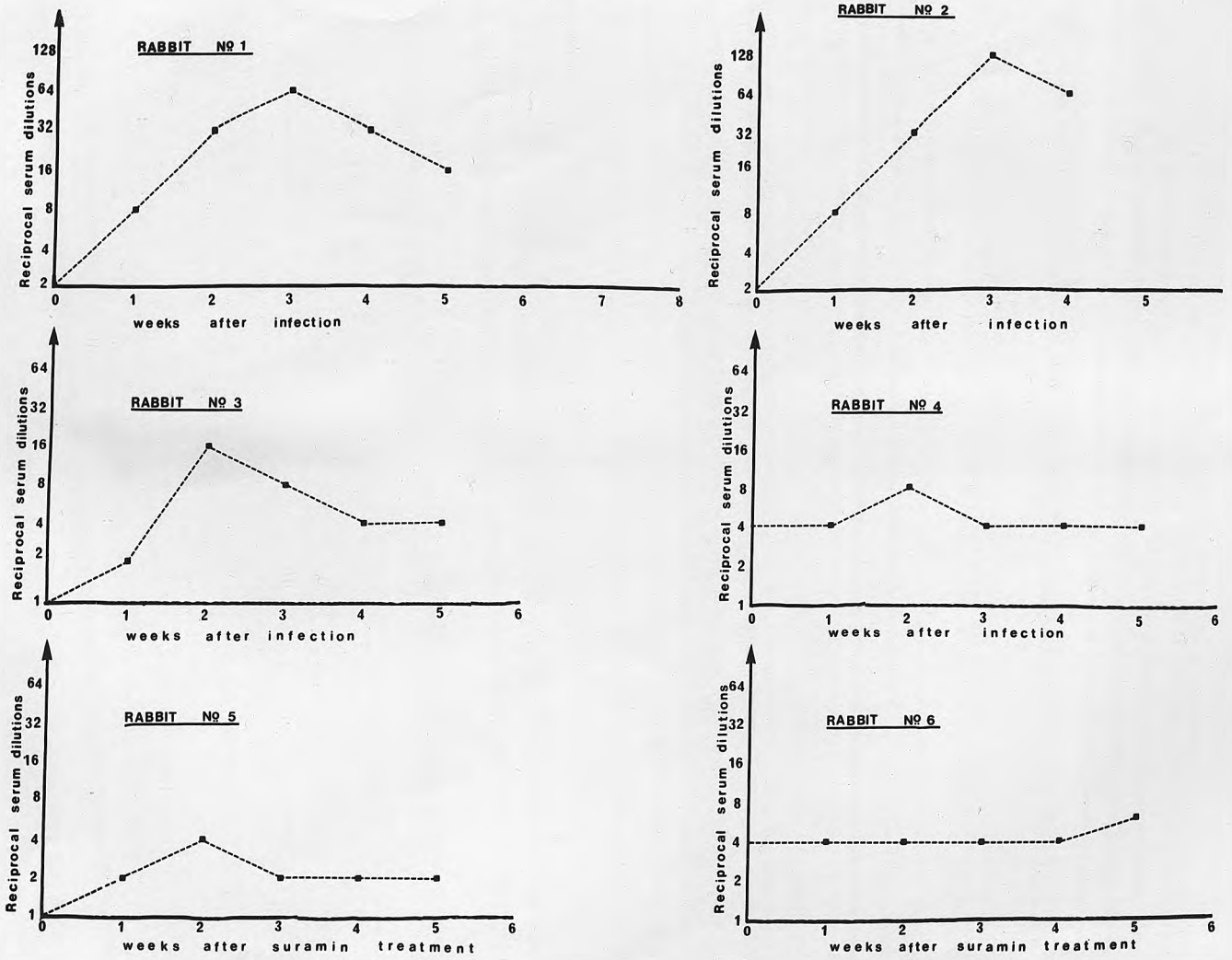

IgG TITRE OF RABBIT SERA
RABBIT Nº 1
RABBIT Nº 2
RABBIT Nº 3
RABBIT Nº 4
RABBIT Nº 5
RABBIT Nº 6
Reciprocal serum dilutions
4096
2048
1024
512
256
128
64
32
0 1 2 3 4 5
weeks
after infection
Died
SURAMIN
after suramin treatment

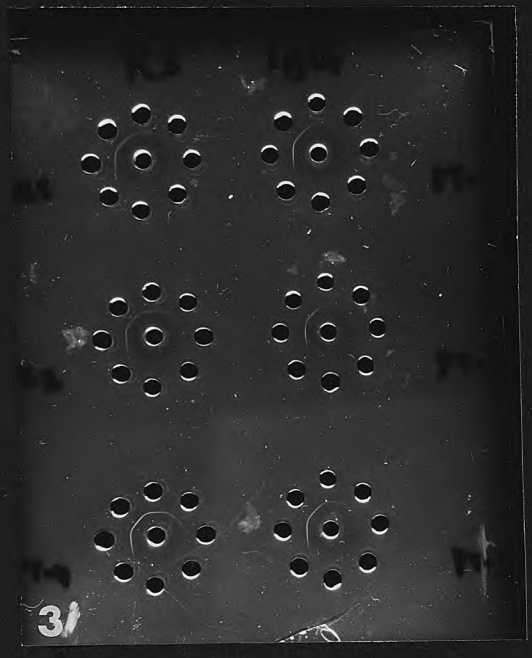
Fig. 5

IgM TITRES OF RABBIT SERA



RABBIT 1 IgG

RABBIT 1 IgM



RABBIT 5 IgG

RABBIT 5 IgM

Plates 1-4. The titration of IgG and IgM by the double diffusion method.

Plates 1 and 2: Titration of IgG and IgM in sera obtained from Rabbit No. 1, before and at week 1-5 after infection with T. brucei.
Plates 3 and 4: Titration of IgG and IgM in sera obtained from Rabbit No. 5, before and after treatment with suramin.

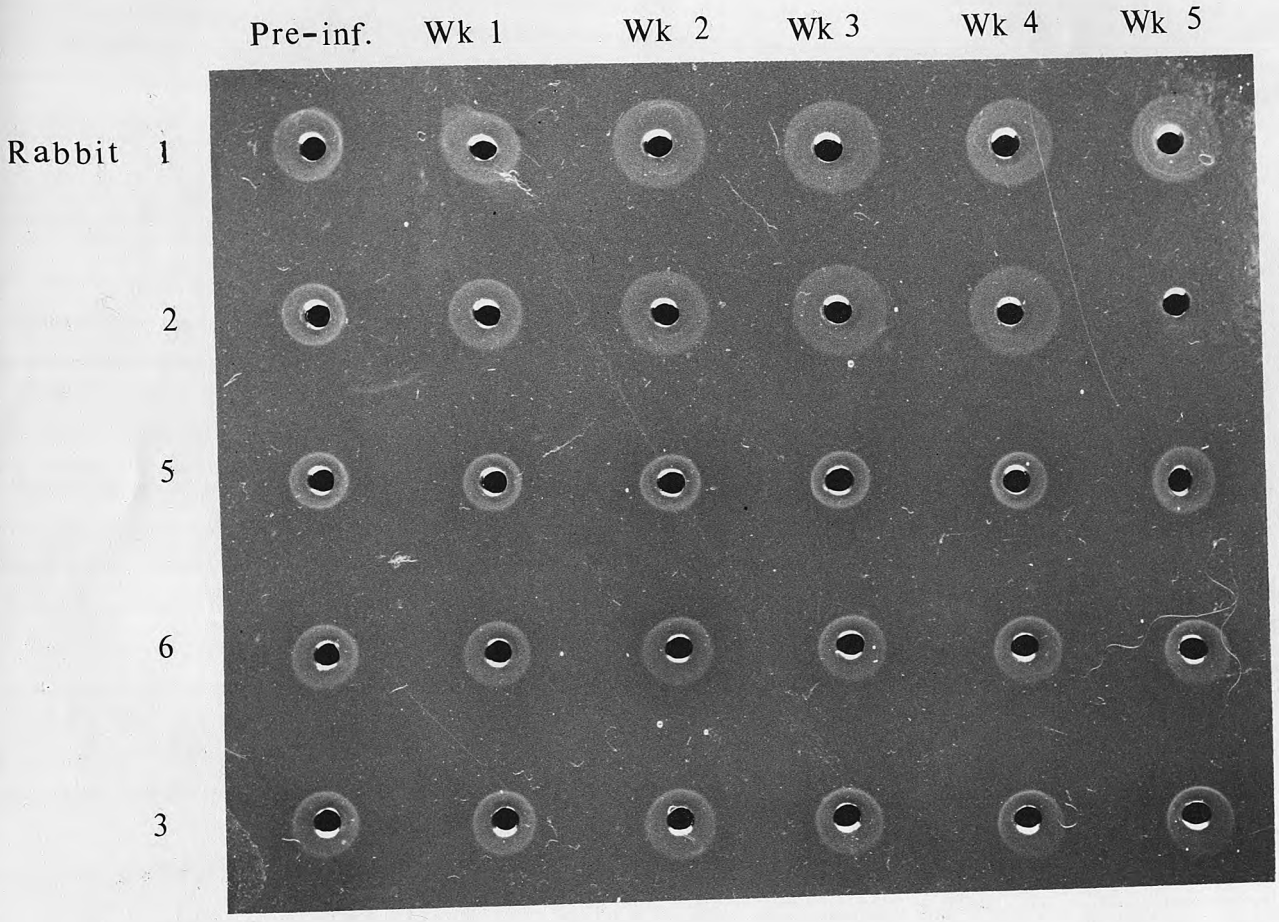


Plate 5. IgM titration in rabbit sera by the single radial diffusion technique, showing an increase in the diameter of the precipitin rings around wells containing sera obtained from Rabbits 1 and 2 during infection with T. brucei TREU 1168.

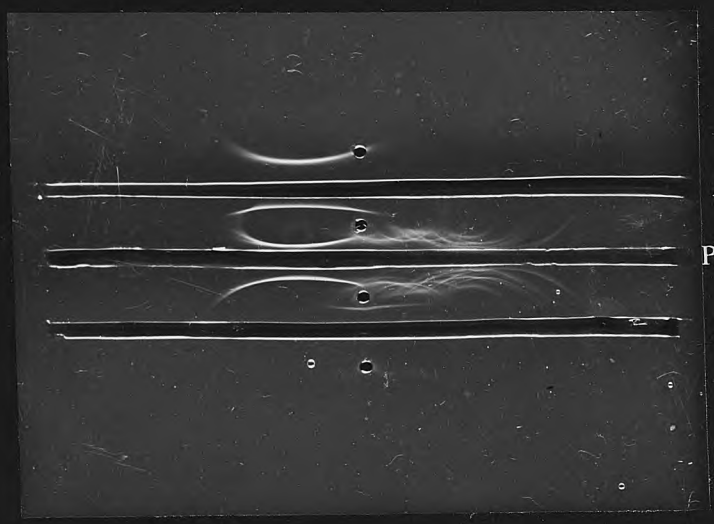


Plate 6 Immunoelectrophoretic picture of normal and infected rabbit sera.

A. Normal rabbit serum against:
   i) Anti-rabbit IgG (Top trough)
   ii) Polyvalent anti-rabbit serum (middle trough)
   iii) Anti-rabbit IgM (Bottom trough)

B. Infected rabbit serum against:
   i) Polyvalent anti-rabbit serum (Top trough)
   ii) Anti-rabbit IgG (Middle trough)
   iii) Anti-rabbit IgM (Bottom trough)

An intense IgM band indicating an elevated IgM level in the infected rabbit serum can be seen.

titre of the pure IgM fractions of the corresponding serum samples from Rabbit 1 were [redacted] 1/640, 1/80 and 1/40 respectively (Table 9). For Rabbit 2, the IgG fraction of serum obtained 4 weeks after infection had an agglutinin titre of 1/80 but the IgM fractions of sera obtained in the 2nd, 3rd and 4th weeks after infection had titres of 1/80, 1/160 and 1/40 respectively (Table 9). Since no agglutinating antibody activity was present in the sera of Rabbits 3, 4, 5, and 6, no agglutination test was carried out with pure immunoglobulin fractions from these animals.

Experiment 2: Comparative Study of immunoglobulins in normal and immunized mice.

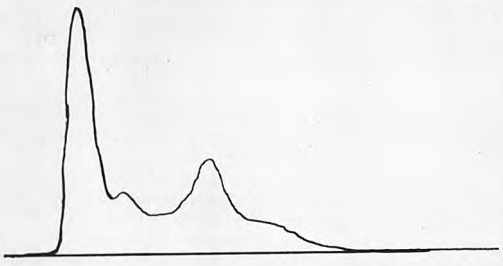
Table 11 shows the total protein content and the relative and absolute concentrations of the serum proteins of mice immunized by infection and drug-treatment and the drug-treatment control group. In the immunized group, the proportion of albumin fell from a preinfection level of 58% to 44% 5 weeks after infection. During the same period, the level of beta-globulins increased from 23% to 30% while the gamma-globulin level reached 10% of the total proteins by the 3rd week after immunization from a preinfection level of 5%. The alpha-globulin content, however, did not show any marked change. The increase in the beta- and gamma-globulin peaks of the electrophoretic patterns of sera obtained from these mice before and at 1-5 weeks after immunization, can be seen in the Chart recordings shown in Fig. 6. In the drug-treated control group, there were no marked changes either in the total protein content or the amounts of the different serum proteins in the sera of the animals during the experiments.

The gamma-globulin content and the agglutinin titres of whole sera and pure immunoglobulin fractions of selected sera against T. brucei

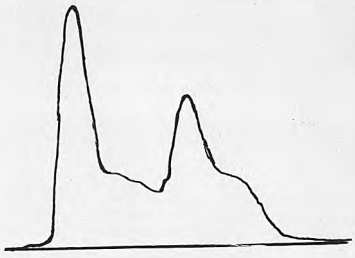
Table 11. The total protein content, relative and absolute concentrations of serum protein fractions of sera from mice immunized against T.brucei TREU 1168 by infection and treatment and drug-treated control mice.

GROUP	SERUM SAMPLE	TOTAL PROTEIN g/100 ml	ALBUMIN		ALPHA-GLOBULIN		BETA-GLOBULIN		GAMMA-GLOBULIN	
			Proportion %	Absolute concentration g/100 ml	Proportion %	Absolute concentration g/100 ml	Proportion %	Absolute concentration g/100 ml	Proportion %	Absolute concentration g/100 ml
A	Preinfection	5·6	58	3·2	14	0·7	23	1·29	5	0·2
	Week 1	8·5	48	4·1	15	1·2	28	2·38	9	0·7
	Week 2	6·5	52	3·4	14	0·9	26	1·69	8	0·5
	Week 3	5·4	53	2·8	16	0·8	21	1·13	10	0·5
	Week 4	5·4	54	2·9	15	0·8	23	1·24	8	0·4
	Week 5	5·3	44	2·3	18	0·9	30	1·59	8	0·4
B	Pretreatment	5·6	58	3·2	14	0·7	23	1·29	5	0·2
	Week 1	3·8	60	2·2	12	0·4	22	0·83	6	0·2
	Week 2	3·8	60	2·3	13	0·4	22	0·83	5	0·1
	Week 3	7·6	60	4·5	13	0·9	21	1·59	6	0·4
	Week 4	4·8	58	2·7	13	0·6	22	1·05	7	0·3
	Week 5	5·9	54	3·1	14	0·8	25	1·47	7	0·4

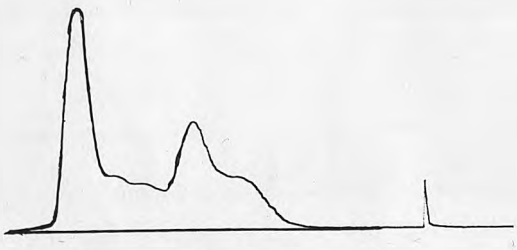
MICE GROUP A



PREINFECTION

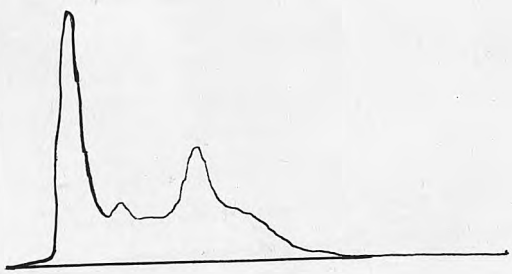


1 WK POST-INF.

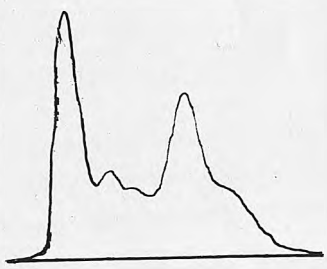


2 WKS POST-INF.

3 WKS POST-INF.



4 WKS POST-INF.



5 WKS POST-INF

Fig. 6. Electrophoretic patterns of sera from mice immunized by infection with T. brucei TREU 1168 followed by treatment with diminazene aceturate.
Note the increase in both the beta- and gamma-globulin peaks.

TREU 1168 are shown in Table 12. Although the absolute concentration of the gamma-globulins in the sera of the immunized group did not show any marked increase, the agglutinin titres of sera from these mice rose to a peak of 1/10240 by the 3rd week after immunization, afterwards declining to 1/80 at 5 weeks after immunization.

The IgM fractions obtained at weeks 1 and 2 after immunization had agglutinin titres of 1/160 but the IgM fraction in the 4th week had a titre of 1/40. No agglutinin activity was present in the IgG fraction of the 2nd week serum sample but that of the 4th week sample showed activity at a dilution of 1/80. No agglutination tests were carried out with immunoglobulin fractions of sera from the drug-treated control mice since whole sera from these did not possess any agglutinin activity.

The results of the challenge experiment to test the immunity of mice treated in the same way as those from which the sera examined above were obtained are shown in Table 13. In subgroup A1, 2 of the 5 immunized mice died 11 and 16 days after challenge while all 5 in subgroup A2 survived for 31 days. In the non-immunized control groups, 4 of the 5 mice in subgroup B1 died 4 days after challenge and the remaining mouse died on the 5th day. Two of the mice in subgroup B2 died on the 5th day while the remaining 3 died on the 6th day after challenge.

Table 12. The gamma-globulin content of the sera of immunized mice and drug-treated controls and the agglutinin titre of whole sera and pure IgG and IgM fractions of selected sera from the immunized group.

GROUP	SERUM SAMPLE	GAMMA-GLOBULIN		AGGLUTININ TITRE		
		CALCULATION g/100 ml	ZINC SULPHATE g/100 ml	Whole Serum	IgG Fraction	IgM Fraction
A	Preinfection	0·2	0·26	Negative		
	Week 1	0·7	0·18	1/80	*ND	1/160
	Week 2	0·5	0·18	1/2560	--	1/160
	Week 3	0·5	0·22	1/10240		
	Week 4	0·4	0·26	1/160	1/80	1/40
	Week 5	0·4	0·22	1/80		
B	Pretreatment	0·2	0·26	Negative		
	Week 1	0·2	0·10	"		
	Week 2	0·1	0·18	"		
	Week 3	0·4	0·15	"		
	Week 4	0·3	0·15	"		
	Week 5	0·4	0·26	"		

*ND Not done.

Table 13. Showing the effect of homologous trypanosomal challenge on mice immunized by infection with *T.brucei* TREU 1168 and drug treatment.

GROUP	SUB-GROUP	NO. OF MICE	CHALLENGE DOSE NO. OF TRYPS.	SURVIVAL PERIOD
A (immunized)	A1	1	19×10^5	11 Days
		2	"	16 "
		3	"	31 "
		4	"	31 "
		5	"	31 "
	A2	1	19×10^3	31 Days
		2	"	31 "
		3	"	31 "
		4	"	31 "
		5	"	31 "
B (Drug-treated Controls)	B1	1	19×10^5	4 Days
		2	"	4 "
		3	"	4 "
		4	"	4 "
		5	"	5 "
	B2	1	19×10^3	5 Days
		2	"	5 "
		3	"	6 "
		4	"	6 "
		5	"	6 "

DISCUSSION

The techniques selected for use in this comparative study of immunoglobulins of normal, infected and immunized animals were in general suitable and yielded useful information. By a combination of electrophoresis, biuret and zinc sulphate tests, it was possible to show serum protein changes in the infected rabbits and immunized mice while the gel diffusion techniques demonstrated an increase in the IgG and IgM levels of these animals. The agglutination and protection tests were used to characterize the antibodies on the basis of their functions. However, more sophisticated methods are needed to analyse the individual immunoglobulins. It was not possible to determine the IgG and IgM levels of mice sera because of the unavailability of mouse antisera. The high cost of antisera and the fact that they are not always readily available, impose limitations on the usefulness of the gel diffusion techniques for measuring immunoglobulins.

The tests employed for estimating the total protein, and the proportions and absolute concentrations of the serum proteins, yielded results which are comparable with those of other workers. The serum protein values of normal rabbits and mice obtained by other workers are given in Table 14 for comparison with the mean values obtained in the present work. The figures show that the tests employed were reasonably accurate and were suitable for monitoring serum protein changes in animals.

The results of the comparison of the immunoglobulins of normal and infected rabbits have shown that trypanosome infection in this species is characterised by a marked increase in the IgM levels as well as an increase in the IgG levels. Similar marked increases in IgG and IgM levels have been found in rabbits infected with T. gambiense (Seed et al., 1969). However, it is now known that much of this

Table 14. Serum protein values of normal rabbits and mice.

	TOTAL PROTEIN g/100 ml	ALBUMIN %	ALBUMIN conc'n	ALPHA-GLOBULIN %	ALPHA-GLOBULIN conc'n	BETA-GLOBULIN %	BETA-GLOBULIN conc'n	GAMMA-GLOBULIN %	GAMMA-GLOBULIN conc'n	REFERENCE
RABBIT	7·2	57·22	4·11	14·94	1·08	14·58	1·06	13·22	0·95	*Hudgins et al., 1956.
		64		13		12·77		9·91		*Allen & Watson, 1958.
		62·0		16·6		9·6		11·8		*Infar, 1967.
	5·01		2·76		1·7		0·29		0·36	Purvis, 1971.
	7·2	68·0		6·0		13·0		13·0		**MacKay, M.
		59·6		7·5		12		21·3		**Stevenson, H.
		63·3		11·5		13·0		4·3		**Deutsch & Goodle.
	5·83	63·3	3·63	10·9	0·61	17·6	0·98	9·4	0·44	Touray.
MOUSE		53·0		14·0		19·0		14·0		*Bucker, 1961.
	3·99		3·38		0·092		0·46		0·058	*Gleeson & Friedberg, 1953.
	5·6	58	3·2	14	0·7	23	1·2	5	0·2	Touray

* From Kaneko, J.J. and Cornelius, C.E. (1970)

** From Long, C. (1961)

immunoglobulins is non-specific. For example, Houba et al. (1969) found no significant decrease in IgM levels in the serum of monkeys infected with T. brucei after absorption with trypanosomal antigen while Freeman et al., (1970) have shown that the non-trypanosome component of IgG in the Rhesus monkey may be as much as 95%. More recently, Mackenzie et al. (1972), and Mackenzie and Boreham (1974) have shown that part of the elevated IgM levels is due to naturally occurring tissue-autoantibodies.

Although the IgM levels in the infected rabbits increased to 16 times the preinfection level within 2 weeks, the increases in the IgG levels were less dramatic and gradual. It is possible that a greater increase in the level of IgG occurs much later in the infection as has been found in T. vivax infection by Clarkson and Penhale (1973).

The serum protein alterations found in Rabbits 1 and 2 were similar to those found in monkeys infected with T. rhodesiense by Woodruff (1959) and in monkeys infected with T. gambiense by Smithers and Terry (1959). Desowitz (1959, 1960) also found similar changes in cattle and antelopes. In the present work, it was found that the increase in the proportion of gamma-globulin during the infection was reflected in the absolute concentration of this serum protein but the decrease in the relative proportion of albumin was not accompanied by any marked fall in its absolute concentration. This would suggest that trypanosome infection has no direct effect on albumin synthesis which is independent of gamma-globulin synthesis, and that any fall in the proportion of albumin is only relative to other serum proteins.

The appearance of high agglutinin titres in the infected rabbits within a week, together with an early increase in the IgM levels,

suggests that agglutinating antibodies play an important role in the defence mechanism against trypanosomes. This is supported by the finding that early in the infection, agglutinating activity (Seed et al., 1969) and protective activity (Seed, 1972) occurred in the IgM fraction. It seems likely that neutralizing antibodies, which Cunningham et al. (1963) found on the 6th day of T. brucei infection in rats,, and the appearance of which coincided with the increase in IgM levels in T. congolense infection in cattle (Luckins, 1974), may be the same as agglutinating antibodies. The fact that both types of antibodies appear early in the infection and reach a peak at about the same time (e.g. the peak agglutinin titre in Rabbits 1 and 2 occurred at 3 weeks while Cunningham et al. (1963) found that neutralizing antibodies reached a peak in 20-30 days in T. congolense - infected cattle) seem to support the view that they might be the same.

It is interesting to note that the immunological response, as judged by the IgG and IgM titres of sera from the rabbits, was more marked in Rabbit 2 which succumbed to the infection in the 4th week. It may be that the higher antibody levels in this rabbit were simply due to the production of non-specific antibodies such as tissue autoantibodies found by Mackenzie and Boreham (1974). On the other hand, it is also possible that the comparatively enhanced immunological response may have exhausted the immunological apparatus of the animal or that the serere response may have aggravated the other effects of the disease, thereby contributing to the cause of death.

The absence of typical serum protein changes and antibody response in Rabbits 3 and 4 was an indication that the attempt to immunize these rabbits by infection and drug-treatment was unsuccessful. The most likely reason for the failure was that the infection did

not establish in the rabbits. This was true in the case of Rabbit 4 in which infection could not be proved by the demonstration of organisms by the examination of stained blood films, wet films, and the buffy coat in the haemotocrit tubes or by mouse inoculation. In the case of Rabbit 3, however, blood collected 1 week after inoculation of parasitaemic blood was infective to one of 3 mice, indicating that the infection was very light at this stage. Because the rabbit was treated at this stage, it was possible that the animal did not experience sufficient antigenic stimulation to produce a detectable immunological response. The lack of agglutinin activity in the sera of this rabbit against T. brucei TREU 1168, which was used to infect the donor rabbit, was probably due to the fact that antigenic variation had already occurred in the donor rabbit at the time the sub-inoculation was carried out (12 days after infection). This would mean that agglutinating antibodies that might have been produced in Rabbit 3, would be against the variant that was present in the donor rabbit at the time of sub-inoculation. Since agglutinating antibodies are variant-specific, such antibodies produced against new variants will not affect the parent strain.

The fact that the drug-treated control rabbits (Rabbits 5 and 6) did not show any changes was an indication that the drug used did not have any effect either on the serum proteins or the antibody response.

Agglutination tests with IgG and IgM fractions of sera selected either to cover the period of the experiment, or on the basis of the quantity available for fractionation, showed that early in the infection, agglutinin activity occurred only in the IgM fraction. This is in agreement with the finding of Seed et al. (1969). Agglutinating activity first appeared in the IgG fraction 4 weeks after infection.

Having demonstrated the purity of the fractions by immunoelectrophoresis and since agglutinating antibodies are trypanosome-specific, it is unlikely that the agglutinating activity in the IgG fractions was due to non-specific antibodies. The very low titres of the pure IgM fractions as compared with those of the whole sera from which they were obtained, may be due to the diluting effect of the buffer and the loss of some of the fractions in the tubes not harvested.

The comparative study of immunoglobulins of normal and immunized mice sera yielded results which extend and corroborate the results obtained in the study of normal and infected rabbit sera. The challenge experiment showed that the attempt to immunize the mice by infection and drug-treatment was successful. Therefore, the mice immunized by this method were a good source of immune serum for the comparative study.

The serum protein changes in the immunized mice were similar to those found in the infected rabbits, except that in mice, the beta-globulins also increased markedly after immunization. The increase in the beta-globulins is not surprising since antibodies also occur in these serum proteins (Humphrey and White, 1970).

The quantitation of IgG and IgM of the sera from the mice could not be undertaken due to unavailability of antisera to these mouse proteins, but Clarkson (1974) found that in T. brucei and T. congolense - infected mice, IgM increased to about 6 times 8 days after imfection and then fell.

The agglutinin titre of the sera from the mice increased more gradually than it did in the infected rabbits probably because of the greater and more prolonged antigenic stimulation in the untreated infection in the rabbits. It is probably for the same reason that higher agglutinin titres were maintained in the rabbits for a longer time than in the mice. As in the rabbits, agglutinin activity occurred in the IgM fraction only up to about 4 weeks after immunization

when the IgG fraction also possessed some activity. The difference between the titre of the pure IgM fraction of the sera obtained from the immunized mice 1 week after immunization and that of the whole serum may be due to the fact that the tests were performed on different days. Although there are many published accounts of the production of antibodies by mice infected with trypanosomes and treated with therapeutic drugs (see Gray, 1967; Herbert and Lumsden, 1968; Lumsden et al., 1973) or immunized by other methods (see Soltys, 1965) there have apparently been no studies other than the present work in which modern methods of characterizing immunoglobulins have been used to relate antitrypanosomal activity of immunized mouse serum to particular immunoglobulin classes. Further work is therefore required to evaluate this aspect of the results of the present study.

SUMMARY

The immunoglobulins of normal rabbits and mice, and rabbits and mice infected and immunized with T. brucei have been compared. The methods used in the study included the biuret test for total protein estimation, zinc sulphate test for gamma-globulin determination and gel diffusion techniques for determining serum immunoglobulin levels. The proportions of the different serum protein fractions were obtained by electrophoresis while characterization of the immunoglobulins was done by gel chromatography and agglutination tests.

In infected rabbits, the total protein content increased during the infection and there was a decrease in the proportion of albumin while the gamma-globulins increased both in proportion and absolute concentration.

The serum IgG titre increased slightly, but the IgM titre increased markedly to more than 32 times the preinfection level within 3 weeks. The agglutinin titre of the sera rose to a peak at 3 weeks but started to decline by the fifth week. Agglutinin activity was found in the IgM fraction alone, up to 3 weeks after infection but thereafter it occurred in both IgG and IgM fractions. Attempts to immunize rabbits by infection and drug-treatment were unsuccessful and no changes occurred in the sera of test and control animals.

In mice, homologous challenge with the infecting strain of T. brucei showed that the immunization procedure was more successful and also demonstrated the protective effect of antibody. The changes in the total serum protein contents were not marked but the relative proportion of albumin in the sera of immunized mice decreased after immunization, while both the beta- and gamma-globulins increased in proportion and absolute amounts. High agglutinin titres appeared within a week and reached a peak at 3 weeks. Agglutinin activity occurred in the IgM fraction only soon after immunization but by the fourth week, both IgG and IgM fractions contained agglutinating and antibodies.

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APPENDIX

Composition of Solutions

Locke's Solution.

Sodium Chloride	9 g.
Calcium Chloride	0.2 g.
Potassium Chloride	0.4 g.
Sodium bicarbonate	0.2 g.
Glucose	2.5 g.
Distilled water to	1,000 ml.

Barbitone acetate buffer. pH 8.6, ionic strength 0.05. For immunoelectrophoresis.

Sodium acetate	6.5 g.
Sodium barbitone	8.87 g.
Barbituric acid	1.13 g.
Distilled water to	2 litres (Gives 50% Solution of reference).

Barbital buffer pH 8.6 ionic Strength 0.075. For electrophoresis.

Diethyl-barbituric acid	0.331 g.
Sodium diethyl barbiturate	1.848 g.
Distilled water	120 ml.

Phosphate buffer pH 8.0.

Stock Solution:

A). 0.154 M.	$Na_2H_2PO_4\ 2H_2O$	20.03 g.
	Distilled water	1,000 ml.
B) 0.103 M.	$Na_2HPO_4\ 12H_2O$	36.89 g.
	Distilled water	1,000 ml.

Add stock solutions in the following proportions to give pH 8.0

Stock Solution A	0.36 ml.
" " B	9.64 ml.

Check pH with pH meter and adjust if necessary.

Borate buffer pH 8.0

Stock Solutions

A) 0.110 M. $Na_2B_4O_7$ $10H_2O$ 41.95 g./litre

B) 0.310 M. H_3BO_3 19.09 g./litre

Add stock solutions in the following proportions to give pH 8.0

Stock Solution A 2.31 ml.

" " B 7.69 ml.

Check pH with pH meter and adjust if necessary.

Solution A. (After Lumsden et al., 1973)

Stock Solutions

Sodium Chloride 0.154 M, 9.00 g./litre

Potassium Chloride 0.154 M, 11.48 g./litre

Magnesium Chloride 0.103 M, 20.94 g./litre

Calcium Chloride 0.103 M, 22.56 g./litre

Mix stock solutions by volume as follows:-

NaCl.	100 volumes
KCl	4
$MgCl_2$	3
$CaCl_2$	1

Solution ABB-8.0 For agglutination test

Mix by volume:

Solution A	9 parts
Borate buffer, pH 8.0	1 part

Solution ABP - 8.0

Mix by volume:

Solution A	9 parts
Phosphate buffer, pH 8.0	1 part.

0.1 M. Tris HCl buffer containing 1 M Na Cl.

0.1 M Tris	12.11 g./litre
1 M NaCl	58.88 g./litre

Adjust pH to 8.0 with 1 N. HCl

Add 0.02% Sodium azide.