

DETECTION OF *COWDRIA RUMINANTIUM* ANTIGEN AND ANTIBODY DURING THE COURSE OF HEARTWATER DISEASE IN SHEEP BY MEANS OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY

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ABSTRACT

NEITZ, A. W. H., VILJOEN, G. J., BEZUIDENHOUT, J. D., OBEREM, P. T., VISSER, L. & VERMEULEN, N. M. J., 1986. Detection of *Cowdria ruminantium* antigen and antibody during the course of heartwater disease in sheep by means of an enzyme-linked immunosorbent assay. *Onderstepoort Journal of Veterinary Research*, 53, 205-207 (1986).

An enzyme-linked immunosorbent assay was used to detect *Cowdria ruminantium* antibodies during the course of heartwater disease. IgM antibodies reached a maximum on the 4th day after infection and disappeared on the 7th day. IgG antibodies first appeared on the 8th day and continued to increase during the remainder of the observation period of 28 days.

The presence of *C. ruminantium* in the blood fractions of diseased animals was demonstrated by an enzyme-linked immunosorbent assay. The earliest day of *C. ruminantium* antigen detection was in plasma and serum on the 4th day after inoculation. Of all the blood fractions investigated, the red blood cells showed the highest concentration, and this reached a maximum on the 12th day after infection.

INTRODUCTION

The duration or nature of the immunity to heartwater disease has not been clarified. It has been reported to vary in sheep from 7-34 months after recovery (Neitz, 1939) or up to at least 4 years if sheep were challenged within 2 months of recovery (Neitz, Alexander & Adelaide, 1947). Immunity appears to abate earlier in cattle than in sheep (Haig, 1955).

Regarding the nature of the immunity, it is not presently known whether the immunity is dependent on the persistence of *Cowdria* in the host (premunition) or not (sterile immunity) (Neitz *et al.*, 1947). Furthermore, it is not known with certainty whether the immunity to heartwater is mediated cellularly or humorally (Alexander, 1931; Du Plessis, 1970). In addition, more information regarding the development of IgG and IgM isotypes during the course of the disease would be valuable in the study of the immunity.

To help clarify some of these aspects, a study was conducted on the development of antibodies and the persistence of the organism in blood fractions of the host during the course of the disease.

Cowdria ruminantium occurs in the blood and vascular endothelial cells of ruminants. In the latter cells, the organisms are found in the cytoplasm as groups, also called colonies or clusters (Cowdry, 1925). Inconsistent results have been reported by various workers regarding the appearance and localization of the organism in various blood fractions. Jackson & Neitz (1932) claimed to have detected isolated organisms free in the blood by light microscopy. Using electron microscopy, Stewart & Howell (1981) and Pienaar (1970) frequently found single organisms in the lumen of capillaries. Cowdry (1925; 1926) believed that single organisms in the blood enter endothelial cells and proliferate to form a colony. This causes the dissemination of the organisms into the blood after cell rupture with commencement of a new cycle. Du Plessis (1975) hypothesized that the initial development takes place mainly in reticuloendothelial cells.

Infectivity tests have implied that the organism is, *inter alia*, located in the white cell fraction (Nemobade,

1976), the red cell fraction (Fawi, Karrar, Obeid & Campbell, 1969), both these fractions (Alexander, 1931) and plasma [Ramisse (1971) cited by Uilenberg (1983)] but not in the serum.

To help explain these conflicting results, an enzyme-linked immunosorbent assay was used in the present study for the detection of the organism in various blood fractions. This may be of value in elucidating the developmental cycle of the organism, the mechanism of invasion of the organism in the host and the pathogenesis of heartwater.

MATERIALS AND METHODS

Analytical quality reagents were used. All glassware and equipment were sterilized with 70 % (v/v) ethanol, and the buffers by filtration through 0,22 μm filters.⁽¹⁾

Investigations into the development of antibodies to Cowdria ruminantium

Preparation of antigen. Antigen was prepared from infected and non-infected *A. hebraeum* nymphae by means of wheat germ lectin Sepharose 6MB (Viljoen, Vermeulen, Oberem, Prozesky, Verschoor, Bezuidenhout, Putterill, Visser & Neitz, 1985). Material eluted in Peak 2 from the column was collected for this purpose.

Preparation of serum. Blood samples (10 ml) of 2 infected and 2 non-infected sheep were taken at 2-7 day intervals during the entire observation period (see below). They were left to clot, and after 4 h the coagulated blood was centrifuged for 10 min at 300 g in a Piccolo bench top centrifuge at room temperature. The sera were siphoned off, divided into 1 ml aliquots and stored at -75 °C in a Specht Scientific deep-freeze.

Detection of antigen in blood fractions

Preparation of blood fractions. Two sheep were injected intravenously (i.v.) with the heartwater-infected nymph suspension (Bezuidenhout, 1981) to obtain heartwater-infected sheep. Two control sheep were injected i.v. with non-infected nymph homogenates. In both cases 0,1 nymph per dose was used. Blood samples (10 ml) were taken in the presence and absence of heparin at 2-7 day intervals subsequent to inoculation (Fig. 2).

Blood samples, collected in heparin, were centrifuged for 10 min at 300 g in a Piccolo bench top centrifuge at 25 °C. The plasma, white blood cells and red blood cells were carefully siphoned off and stored at -75 °C.

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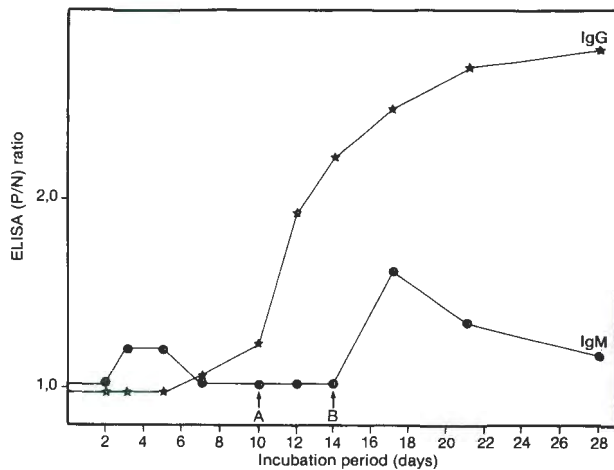


FIG. 1. The detection of antibodies by ELISA to *Cowdria ruminantium* in the serum of heartwater-infected sheep. (A) Commencement of temperature reaction. (B) day of treatment. IgG (**) and IgM (●).

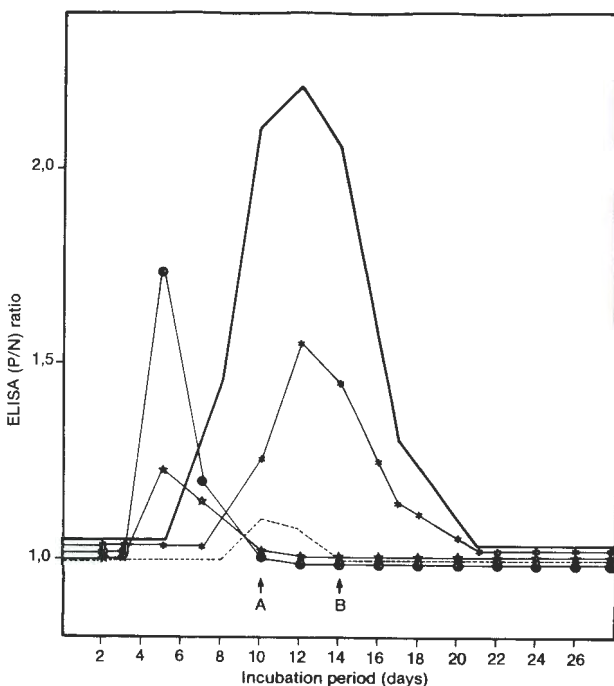


FIG. 2. ELISA screening for *Cowdria ruminantium* antigen in blood fractions of heartwater-infected sheep. (A) Day of reaction, (B) day of treatment. Serum (**), plasma (● ●), whole blood (-----), red blood cells (—) and white blood cells (**).

Samples collected without heparin were left to clot, after which the serum was siphoned off and stored under the same conditions.

Preparation of antiserum. Serum from heartwater-infected and non-infected sheep was prepared as described by Viljoen *et al.* (1985) 4 weeks after the treatment of the diseased animals.

Protein determinations

Protein was determined by the high temperature biuret-Folin method, as described by Dorsey, McDonald & Roels (1977), using bovine serum albumin as standard.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA method described by Neitz, Viljoen, Bezuidenhout, Oberem, Verschoor, Putterill, Visser & Vermeulen, (1986) was used. Serum solutions were made up as follows: 1 ml of serum was diluted 1:30 with 0.05 M Tris, 0.154 M NaCl buffer, pH 7.4. For the

detection of IgM antibodies in serum, a 1 000 × dilution of rabbit-anti sheep IgM peroxidase (μ chain specific)⁽¹⁾ conjugate in 1 % (w/v) bovine serum albumin/phosphate buffered saline was used. As antigens, 10 μ g protein/ml of infected and non-infected blood fractions or Peak 2 wheat germ lectin column, obtained from infected or non-infected nymphae, were used.

The ratios reported for antibody screenings were obtained from infected and control sheep, using Peak 2 obtained from wheat germ lectin affinity chromatography as antigen source.

The infected to non-infected (P/N) ratios reported for the detection of *C. ruminantium* in blood fractions were calculated by dividing the absorbance values obtained for infected animals by the values obtained for the control animals.

RESULTS

In the early stages of the disease, there were insignificant increases in infected to non-infected ratios for IgM and IgG specific for *C. ruminantium* (Fig. 1). IgM antibodies were first observed in small amounts 3 days after the inoculation. Their concentration reached a maximum on the 4th day, after which these immunoglobulins disappeared on the 7th day. IgG antibodies first appeared on the 8th day and continued to increase during the remainder of the observation period of 28 days. Shortly after treatment with oxytetracycline, a sharp rise in the IgM level occurred and this reached a maximum on the 3rd day after treatment. The level subsequently decreased.

The presence of *C. ruminantium* antigen in the blood fractions of heartwater-infected sheep was demonstrated by ELISA (Fig. 2). Plasma, the red blood cell and white blood cell fractions exhibited infected to non-infected ratios higher than 1.5. Day 4 after the inoculation was the earliest day on which *C. ruminantium* antigen was detected in plasma and serum.

In the red blood cell fraction, the antigen was first detected on the 6th day, and in the white blood cell fraction 2 days later. Of all the fractions investigated, erythrocytes showed the highest P/N ratio, and this reached a maximum of 2 days after the commencement of the febrile reaction. Antigen levels declined in the red and white blood cell fractions upon treatment with oxytetracycline and became undetectable on the 7th day after treatment.

DISCUSSION

Significant IgG levels only were detected from the 10th day after inoculation of sheep with *C. ruminantium* infected nymph suspension. The inability to detect more IgM present in the initial stages of the disease could be due to the binding of antigen to IgM resulting in the absence of free IgM in the serum in the initial stages of the immune response. This was also observed by Benacerraf & Gell (1959) in their studies on hypersensitivity. They reported delayed and Arthus-type skin reactivity to protein conjugates in guinea-pigs. This could explain the increase of free IgM upon treatment of the disease with oxytetracycline, which inhibited proliferation of the organism.

It is evident from the results obtained on the localization of *C. ruminantium* in the blood of the host that the organism enters the bloodstream and is initially detectable in the plasma and serum. First the erythrocytes and subsequently the leukocytes are extensively invaded, with a concomitant decrease of *C. ruminantium* levels in plasma and serum. On Day 21, i.e. 7 days after treatment of the diseased animals, no *C. ruminantium* antigen

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was detectable in any of the blood fractions. The low P/N ratios observed in whole blood is probably due to the small amount of *C. ruminantium* antigen relative to the total blood protein concentration.

It appears that the detection of *C. ruminantium* antigen, or antibodies against it, in blood by an ELISA should be useful for the diagnosis of the disease. This method is suitable not only for identifying the aetiological agent in blood in a highly specific manner but also for monitoring the time course of the immune response. In this way the change in antibody titre, i.e. the appearance of antibody and its subsequent decline, may be established.

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