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THEORETICAL ASPECTS OF THE ENZYME-LINKED IMMUNOSORBENT ASSAY TECHNIQUE AND ITS USE IN THE DETECTION OF *COWDRIA RUMINANTIUM* ANTIGEN AND ANTIBODY IN REACTING ANIMALS

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ABSTRACT

VILJOEN, G. J., VERMEULEN, N. M. J. & NEITZ, A. W. H., 1987. Theoretical aspects of the enzyme-linked immunosorbent assay technique and its use in the detection of *Cowdria ruminantium* antigen and antibody in reacting animals. *Onderstepoort Journal of Veterinary Research*, 54, 305–312 (1987).

Numerous parameters affect the enzyme-linked immunosorbent assay activity and the assay conditions must therefore be carefully controlled to obtain reproducible results. These parameters include temperature, pH, ionic strength, buffer composition, cofactors, substrate depletion, product inhibition, increasing reversal of reaction as product concentration increases, adsorption of enzyme to solid supports, denaturation and non-enzymatic background rate.

An ELISA 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 disappeard 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 detection of *C. ruminantium* antigen was in plasma and serum on the 4th day after inoculation. Of all the blood fractions investigated, the red blood cell fraction showed the highest concentration and this reached a maximum on the 12th day after infection.

THEORETICAL ASPECTS OF THE ENZYME-LINKED IMMUNOSORBENT ASSAY

The time-course of an immune response in infectious disease has been shown to be different when measured with different antigenic preparations and different assays. For example: in brucellosis agglutination, titres appear early in infection and persist at low levels for years, whereas precipitin levels appear later and disappear much sooner. In many rickettsial diseases the antibody titres determined by complement fixation appear later and subside sooner than those measured by neutralization (Davis *et al.*, 1968).

Some of these diversities may be explained by the following possibilities: 1. A given organism is an assembly of numerous antigens or antigenic determinants which differ in immunogenicity and stability in the host. 2. There are functional and structural differences in the antibodies specific for a given antigenic determinant and different antibody isotypes are formed at different stages of the immune response. For example IgM antibodies are formed early in the response and are especially effective in the agglutination assays. 3. There are differences in the various serological methods and some assays may thus detect antibody earlier and more persistently than others.

Enzyme-linked immunoassays offer several advantages, notably high sensitivity and the possibility of handling numerous samples simultaneously. Processors which perform automatically and precisely most of the time-consuming and technically arduous procedures of the assay are commercially available (Goodburn, Williams & Marks, 1982). The assay is also not subject to observer bias (Engvall, Jonsson & Perlmann, 1971; Voller, Bartlett & Bidwell, 1978; Clark & Engvall, 1983; Voller, 1983; Blake & Gould, 1984). By incorporating appropriate controls a high degree of specificity can be obtained.

Since enzymes act as catalysts they convert many moles of substrate to product by repeating the catalytic event over and over. They thus act as amplifiers and by employing enzymes with high turnover numbers (up to 10^7 mole of substrate to product/mole of enzyme/min.), as indicators, extremely low concentrations of antibody or antigen can be detected (Maggio, 1983).

Numerous parameters affect the measurement of enzyme activity and the assay conditions must therefore be carefully controlled to obtain reproducible results (Maggio, 1983). These parameters include temperature, pH, ionic strength, buffer composition, cofactors, substrate depletion, product inhibition, increasing reversal of reaction as product concentration increases, adsorption of enzyme to solid supports, denaturation and nonenzymatic background rate.

Enzymes suitable for the ELISA should meet several criteria: 1. Possess a high turnover number, low K_{ms}, high K_{mp}, a pH optimum compatible with good antigen-antibody association. 2. Have an easily detectable product. 3. Have long-term stability. 4. Have high retention of activity after coupling. 5. Have minimal effect on antibody-antigen association. 6. Should not be present in biological samples to be assayed (e.g. should ideally not be an enzyme present in serum) and be readily available (Maggio, 1983).

Noteworthy are the requirements for low K_{ms} and high K_{mp} values. This is evident from the following considerations: The catalytic process involving an enzyme by which substrate (S), is converted to product (P) entails an enzyme-substrate complex (ES) (equation 1).

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} E+P \qquad \ldots\ldots\ldots\ldots (1)$$

The innitial velocity for the reaction in each direction may be written as (Segel, 1975):

$$v_f = \frac{V_{maxf}[S]}{K_{ms} + [S]} \qquad \ldots\ldots\ldots\ldots (2)$$

(in the absence of P), and

$$v_r = \frac{V_{maxr}[P]}{K_{mp} + [P]} \qquad \ldots\ldots\ldots\ldots (3)$$

(in the absence of S)

where V_{maxf} and V_{maxr} denote maximal velocities in the forward and reverse directions and K_{ms} and K_{mp} denote the Michaelis constants for substrate and product respectively (equation 4 and 5).

$$K_{ms} = \frac{k_2 + k_{-1}}{k_1} \qquad \ldots\ldots\ldots\ldots (4)$$

$$K_{mp} = \frac{k_2 + k_{-1}}{k_{-2}} \qquad \ldots\ldots\ldots\ldots (5)$$

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The constant k_2 (or k_{cat}) is the turnover number (Palmer, 1981) (molecular activity or catalytic rate constant) and represents the maximum velocity per mole of enzyme (or per mole of catalytic site).

$$k_2 = \frac{V_{maxf}}{[E]_t} \quad \ldots\ldots\ldots\ldots (6)$$

The k_2 and K_{ms} values are usually in the range of 50–10^7 min^{-1} and 10^{-6} to 10^{-2} M respectively.

It is evident from equation 2 that if $[S] << K_{ms}$, v would be very sensitive to changes in [S] and $v << V_{maxf}$. It thus follows that for ELISA, a $[S] >> K_{ms}$ is required. Under these conditions v is independent of [S] and consequently V_{maxf} is measured which is a measure of $[E]_t$ (equation 6) which in turn is a measure of antibody or antigen concentration. With $[S]/K_{ms}$ values of 1, 10 or 100, equation 2 predicts velocities of 0,5 V_{maxf}; 0,9 V_{maxf} and 0,99 V_{maxf}, respectively. The lower the value of K_{ms}, the lower the [S] required to reach V_{maxf}. This is important with substrates having low solubilities. Furthermore, enzymes with low K_{ms} values are less sensitive to competitive inhibitors (Segel, 1975) which may be present in samples to be assayed.

From equation 3 it is clear that when K_{mp} is large, the reverse reaction has only a minor effect on the observed netto reaction. When K_{mp} is large and V_{maxr} small, the rate of product formation proceeds linearly for the greatest period of time. This minimizes the effect of variations in the time between substrate addition and measurement of product.

It should be borne in mind that the properties of an immobilized enzyme, as used in ELISA may be different from those of the same enzyme in free solution. This is dependent upon the method used for the coupling and the nature of the carrier (antigen or antibody) (Maggio, 1983). Thus the pH optimum, V_{max} and K_m may be affected. The latter is influenced by the electrostatic field of the carrier and by diffusion factors. Enzymes attached to antibodies or antigens may influence the antibody-antigen association resulting in a lower sensitivity.

Numerous methods for coupling enzymes to antibody or antigen are available (Kabakoff, 1983) and suitable enzymes for ELISA have been described (Blake & Gould, 1984). Those most commonly used are peroxidase, alkaline phosphatase and β-galactosidase. These enzymes can be detected at femtomole levels by spectrophotometry. Peroxidase is the cheapest and several chromogens are available that produce dark colours which are suitable for visual determinations. However, many of these compounds have been shown to be carcinogenic or mutagenic. The purity of the enzyme-antibody conjugate is important. The presence of free enzyme may cause high background signals, while free antibody trends to dilute out the antibody conjugate (Blake & Gould, 1984).

Enzyme immunoassays (EIA) may be classified into two groups (Rubenstein, 1978; Clark & Engvall, 1983): 1. The heterogeneous EIA in which the enzyme-labelled antigen or antibody is separated from the enzyme-labelled antigen-antibody complex prior to measurement of enzyme activity, and 2. the homogeneous EIA in which the enzyme activity of labelled antigen is measured in the presence of the labelled antigen-antibody complex, the enzyme moiety of which is sterically inhibited.

The enzyme-linked immunosorbent assay (ELISA) is a heterogeneous EIA (Clark & Engvall, 1983). It may be classified as competitive or noncompetitive depending on whether unlabelled antigen and antigen linked to an enzyme or attatched to a solid phase compete for a limited number of antibody binding sites or whether the antigen or antibody to be measured is allowed to react alone with an excess of immune reactant. The noncompetitive assay offers several advantages and numerous assay configurations have been devised (Voller *et al.*, 1978; Voller, 1983; Clark & Engvall, 1983).

In our study, concerning the ELISA, the approach depicted in Fig. 1 was employed. Advantages of this method include: 1. Incubation of enzyme-labelled antibody with a preformed antibody-antigen complex is separate from the incubation of antibody with antigen, thus reducing possible factors in serum which may influence the enzymatic reaction. It should be noted that the presence of certain serum factors may produce undesirable non-specific effects even in heterogeneous assays (Kato, Umeda, Suzuki & Kosaka, 1980). 2. The enzyme-labelled antibody is available commercially and neither antigen nor specific antibody need be conjugated with enzyme. 3. Amplification of sensitivity is achieved since several enzyme-labelled antibody molecules may bind to a single polyvalent antigen (specific antibody) (Fig. 1).

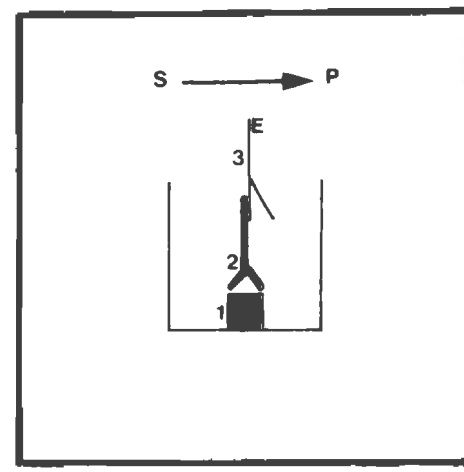

FIG. 1 Schematic presentation of ELISA configuration used for the detection of *C. ruminantium* antigen or antibody in heartwater reacting animals. 1: antigen; 2: specific antibody; 3: enzyme-antibody conjugate (secondary antibody) directed against sheep immunoglobulins.; S = Substrate; P = Product.

Factors which may lead to inconsistent results should be observed when performing an ELISA. An important step in the assay is the immobilization of antigen or antibody. This is usually achieved by physical adsorption to plastic carriers (polystyrene, polyvinyl, polypropylene or polycarbonate). Adsorption of proteins to plastic surfaces is probably due to hydrophobic interaction. The rate and extent of coating is dependent upon the diffusion coefficient of the protein, the ratio of surface area to be coated to the volume of coating solution, the concentration of the adsorbing substance, the temperature and the duration of the adsorption reaction (Clark & Engvall, 1983). It should be borne in mind that the antigen or antibody is not covalently bound to the plastic and loss of adsorbed protein may occur during the incubation and washing procedures. Furthermore, nonuniform adsorption may occur in the wells caused by inhomogeneities in the plastic material (Chessum & Denmark, 1978; Goodburn *et al.*, 1982). In addition adsorption may result in denaturation to a certain extent leading to loss of binding capacity.

The adsorption process is in contrast to the antigen-antibody interaction nonspecific. Thus adsorption of antibody or antigen to the solid phase may occur during the subsequent incubation steps. This can be eliminated by blocking vacant sites on the solid phase with an inert

protein and by incorporating a neutral detergent (Tween 20) which does not interfere significantly with the antigen-antibody reaction. The detergent concentration should be such as to prevent the formation of new hydrophobic interactions but not disrupt existing ones.

Apart from nonspecific adsorption to the solid phase, the removal of free protein in solution may pose a problem. This arises from the negative electric potential at the surface of most plastics resulting in a diffuse double-layer of ions at the interface. Vigorous or prolonged washing is required to displace this double layer (Clark & Engvall, 1983).

Other crucial steps in enzyme-linked immunosorbent assays are the antigen-antibody interactions. In the assay used in the present study two such steps are employed viz, antigen-first antibody interaction and first antibody-anti-first antibody-enzyme conjugate interaction.

For a simple hapten-anti-hapten (Butler, 1983) association the reaction may be presented as follows:

$$Ab + H \underset{k_2}{\overset{k_1}{\rightleftarrows}} Ab - H$$

The intrinsic association constant, K_o is defined as:

$$K_o = \frac{k_1}{k_2} \quad \ldots(7)$$

in which k_1 and k_2 are the association and dissociation rate constants, respectively (Steward, 1977).

The k_1 values have been shown to be within one order of magnitude of 10^8 $M^{-1}s^{-1}$ for all hapten-anti-hapten systems tested. On the other hand, a great variation in the dissociation rate constants have been observed ($3{,}4 \times 10^{-4} - 6 \times 10^3$ s^{-1}). Froese (1968) has suggested that the stability of the hapten-antibody complex is governed by the dissociation rate constant. Further investigations have confirmed that k_2 determines the affinity of antibody for hapten (Steward, 1977). In the case of multivalent antigens the antibody-antigen interaction may be reversible (Steward, 1977). This is of particular importance in heterogeneous ELISA to prevent loss of antibody or antigen during the vigorous washing procedures. It is thus imperative that antibodies with high affinity be employed for ELISA. The obtainment of such antibodies may require much effort since many factors determine their affinity.

Progressive increase in antibody-antigen association constants have been shown to occur with time after immunization (Eisen & Siskind, 1964). This phenomenon has been termed the maturation of the immune response (Siskind & Benacerraf, 1969). Affinity maturation has been shown to be the result of changes in the cell population producing antibody rather than selective removal of high affinity antibody by the excess antigen present early in the response (Steiner & Eisen, 1967). Other temporal changes of affinity variation may occur; an early rise in affinity followed by a fall in affinity and no change in affinity with time (Steward & Steensgaard, 1983).

The changes in affinity of antibody with time after immunization is affected substantially by the nature of the immunogenic stimulus (Steward, 1977). The antigen-depot effect produced by immunization with Freund's complete adjuvant results in a slow release of antigen over extended periods and in sustained maturation of affinity (Steward, 1977). Thus, immunization in saline results in the relatively rapid elimination of the antigen and subsequent termination of stimulation by the antigen (Steward, 1977).

The antigen dose rate is also known to influence the affinity of the antibody produced. Immunization with large doses of antigen in Freund's complete adjuvant results in a reduction in the rate of maturation of affinity compared to when small doses are administered (Eisen & Siskind, 1964). This effect has been ascribed to the stimulation of also low affinity cells in the presence of excess antigen. Further, under such situations it may be possible that the high affinity cells may be rendered tolerant (Steward, 1977).

Apart from the above described time factor, dose rate and nature of the immunogenic stimulus, other factors determine the affinity of antibody. They include genetic and cellular factors, immunological tolerance, antigenic competition (inhibition of the immune response to one antigen by the administration of another antigen), aging and nutritional state (Steward & Steensgaard, 1983). By pooling sera from different animals to obtain antibody preparations for analytical purposes, some of these factors may be averted (Steward & Steensgaard, 1983).

The specificity of the assay is determined principally by the antibody. Specificity of antibody preparations can be increased with immunosorbent affinity chromatography by which interfering antibodies can be removed. However, cross-reaction of antigens with epitopes similar to those on the immunizing antigen limits the specificity. It is therefore evident that it is virtually impossible to certify absolute specificity since infinite antigens need to be tested for possible cross-reaction. In practice however, true specificity can be approached, but not entirely met, by employing monoclonal antibodies for antigen detection (Newmark, 1985) or pure antigen for antibody detection and by employing appropriate controls. The use of control assay in the present study is shown in Fig. 2.

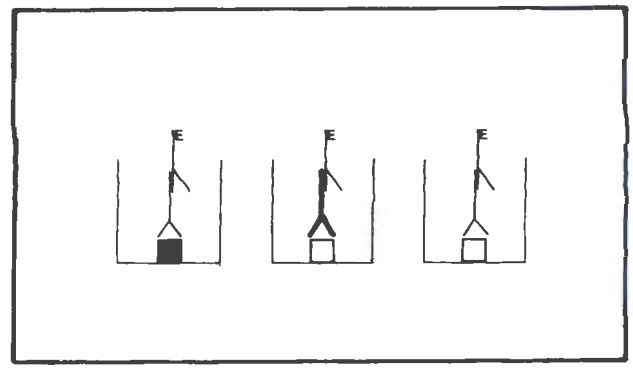

FIG. 2 Schematic presentation of controls used in the ELISA shown in Fig. 1. ■ and □: antigen obtained from heartwater$^+$ and heartwater$^-$ (hw$^+$ and hw$^-$) material respectively, (e.g. hw$^+$ or hw$^-$ nymphae or blood fractions obtained from sheep infected with either hw$^+$ or hw$^-$ nymphae). ⟵ & ⟵ antibody obtained from sheep infected with either hw$^+$ or hw$^-$ nymphae respectively; ⟵E: enzyme-antibody conjugate directed against sheep immunoglobulins.

INTRODUCTION

The detection of *Cowdria ruminantium* antigen and antibody during the course of heartwater disease is important for several reasons. Of prime importance is the diagnosis of the disease in living animals. The diagnosis of infectious diseases is usually established both by identification of the putative causative agent and by demonstrating a change in specific antibody during the course of the infection and convalescence (Davis, Dulbecco, Eisen, Ginsberg & Wood, 1968; Cox, 1981). In addition, the duration (Neitz, 1939; Neitz, Alexander & Adelaar, 1947; Haig, 1955) or nature (Alexander, 1931; Neitz *et al.*, 1947; Du Plessis, 1970) of the immunity to heartwater has not been clarified.

TABLE 1 Immunochemical assays for antigens of chlamydia and rickettsiae-like organisms and their antibodies

Assay	Sensitivity minimum detectable (ng)[a]		References[e]
	Antigen	Antibody	
Primary reactions[c]			
Enzyme-linked immunosorbent assay	0,05	0,05	1, 2, 3, 4, 5, 6, 7
Enzyme-linked fluorescence immuno assay		<<0,05[b]	8
Indirect fluorescent antibody test		75[b]	5, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 27
Secondary reactions[c]			
Weil-Felix test			10, 20
Complement fixation	15	100	10, 13, 15, 16, 17, 18, 19
Counter immunoelectrophoresis	150	1 250	21
Rocket immunoelectrophoresis	5 000[d]	50 000[d]	22
Crossed immunoelectrophoresis	5 000[d]	50 000[d]	22, 23
Flocculation test			24, 25
Radioimmunoprecipitation			2, 26
Passive haemagglutination		10	10, 13, 19
Latex agglutination			10, 13, 14
Microagglutination			13, 18, 19

[a] Data from Butler (1983)

[b] Values depicted from Clausen (1981); Butler (1983); Clark & Engvall (1983)

[c] Primary reactions occur within milliseconds and are macroscopically invisible *per se*. Secondary reactions require a longer time (minutes to hours) and are visible microscopically or to the unaided or partially aided eye (Butler, 1983)

[d] Values recorded for 'immunoelectrophoresis' (Butler, 1983)

[e] *References*

(1) Viljoen, Vermeulen, Oberem, Prozesky, Verschoor, Bezuidenhout, Putterill, Visser & Neitz (1985); (2) Herrmann, Hollingdale, Collins & Vinson (1977); (3) Roges & Edlinger (1986); (4) Behymer, Rupanner, Brooks, Williams & Franti (1985); (5) Clements, Dumler, Fiset, Wisseman, Snyder & Levine (1983); (6) Crum, Hanchalay & Eamsila (1980); (7) Halle, Dasch & Weiss (1977); (8) Numazaki, Chiba, Yamanaka, Moroboshi, Aoki & Nakao (1985); (9) Du Plessis (1981); (10) Kaplan & Schonberger (1986); (11) Newhall, Batteiger & Jones (1982); (12) Treharne, Darougar & Jones (1977); (13) Hechemy, Anacker, Carlo, Fox & Gaafor (1983); (14) Rawlings, Elliot & Little (1985); (15) Philip, Casper, Ormsbee, Peacock & Burgdorfer (1976); (16) Dupuis, Péter, Peacock, Burgdorfer & Haller (1985); (17) Wang & Grayston (1974); (18) Newhouse, Shepard, Redus, Tzianabos & McDade (1979); (19) Philip, Casper, MacCormack, Sexton, Thomas, Anacker, Burgdorfer & Vick (1977); (20) Cox (1981); (21) Caldwell & Kuo (1977); (22) Dasch, Samms & Williams (1981); (23) Caldwell, Kuo & Kenny (1975); (24) Ilemobade & Blotkamp (1976); (25) Ilemobade (1976); (26) Williams, Walker, Peacock & Stewart (1986); (27) Sahu (1986)

Inconsistent results have emerged from several studies on the appearance and localization of the organism in various blood fractions during the course of the disease (Uilenberg, 1983). Clarification of these aspects may be of value in the elucidation of the developmental cycle of the organism, the mechanism of invasion of the organism in the host, and the pathogenesis of heartwater.

Numerous immunological methods have been applied for the detection of antigens of Chlamydia and rickettsiae-like organisms and antibodies against them. These data are summarized in Table 1. It is quite clear from Table 1 that the ELISA technique is an extremely sensitive assay in the detection of antibody or antigen. It is in the order of 500 times more sensitive than the other known methods used in *C. ruminantium* assays.

A non-serological test based on gas-liquid chromatographic analysis of sera has been investigated for early diagnosis of rickettsial disease (McDade, Wells, Brooks & Alley, 1981). This method proved to be non-specific for the diagnosis of specific rickettsial diseases. Several of these tests have been compared with respect to their sensitivity in diagnosis (Philip *et al.*, 1977; Newhouse *et al.*, 1979; Clemments *et al.*, 1983; Rawlings *et al.*, 1985; Kaplan & Shonberger, 1986).

In our hands, the ELISA method has proven to be useful in detecting antigen during the isolation of *Cowdria ruminantium* (Viljoen *et al.*, 1985; Neitz, Viljoen, Bezuidenhout, Oberem, Putterill, Verschoor, Visser & Vermeulen, 1986) and in various tissues of *Amblyomma hebraeum* (Neitz, Viljoen, Bezuidenhout, Oberem, Van Wyngaardt & Vermeulen, 1986; Viljoen, 1985). This paper describes the determination of *C. ruminantium* antigen and antibody in animals with heartwater disease. Parts of this work have been published (Neitz, Viljoen, Bezuidenhout, Oberem, Visser & Vermeulen, 1986).

MATERIALS AND METHODS

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.

Detection of antigen in blood fractions

Preparations of blood fractions. For this study, infected and non-infected antigen were obtained from heartwater$^+$ and heartwater$^-$ (hw$^+$ and hw$^-$) material respectively (e.g. hw$^+$ or hw$^-$ nymphae or blood fractions obtained from sheep infected with either hw$^+$ or hw$^-$ nymphae). Two sheep were injected intravenously (i.v.) with a heartwater-infected *A. hebraeum* nymph suspension (Bezuidenhout, 1981) to obtain heartwater-infected sheep. Two control sheep were injected i.v. with non-infected *A. hebraeum* nymph homogenates. In both cases 0,1 nymph per dose was used. Blood samples (10 mℓ) were taken with or without heparin at 2–7 day intervals subsequent to inoculation (Fig. 3). Reacting sheep were treated with oxytetracycline (Neitz *et al.*, 1986).

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. 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.

Detection 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 6 MB (Viljoen *et al.*, 1985). Since *C. ruminantium* organisms show staining characteristics similar to gram-negative bacteria (Cowdry, 1925) an attempt was made to purify viable *C. ruminantium* organisms by means of lectin affinity chromatography. Wheat germ lectin shows specificity

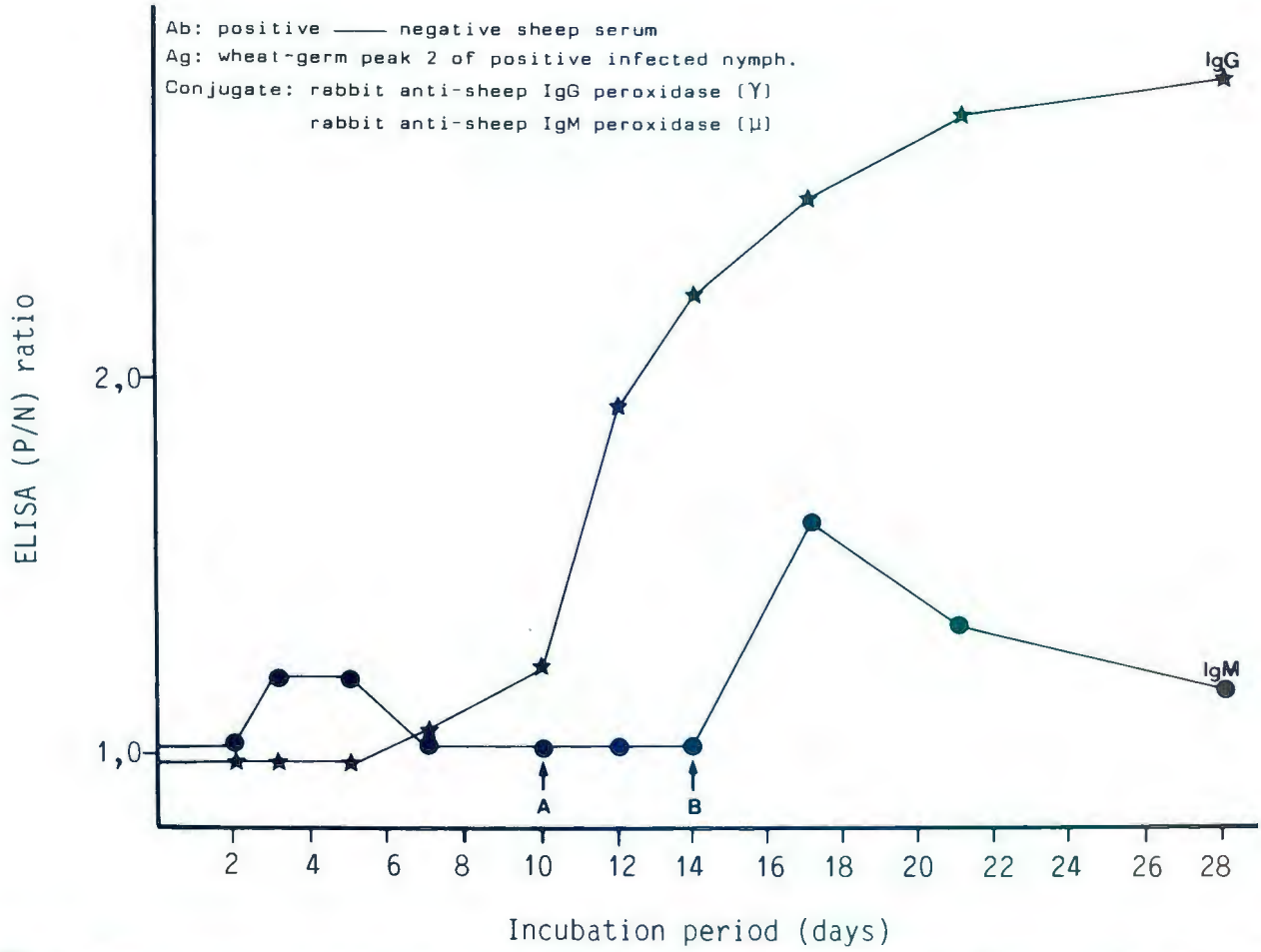

FIG. 3 Detection of antibodies by ELISA to *Cowdria ruminantium* in serum of heartwater-infected sheep. (A) Commencement of temperature reaction. (B) Day of treatment. IgG (★★) and IgM (●●).

towards N-acetyl-D-glucosamine (Nagata & Burger, 1974) which is a characteristic constituent of the cell wall of gram-negative micro-organisms (Salton, 1964). Lectin affinity Chromatography separation is based on the presence of specific sugars of the cell membrane. Material eluted in Peak 2 (bound peak to the column representing the material containing the appropriate sugar) from the column was collected for this purpose.

Collection of serum. Blood samples (10 mℓ) from 2 heartwater infected and 2 heartwater 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.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA method described by Neitz *et al.* (1986) was used. Serum dilutions 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 1:1000 dilution of rabbit-anti sheep IgM peroxidase (μ chain specific) (Cooper Biochemicals) conjugate in 1 % (w/v) bovine serum albumin/phosphate buffered saline was used. As antigens, 10 μg 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

Detection of antibody

In the early stages of the disease, there was an insignificant increase in infected to non-infected ratios IgM and IgG specific for *C. ruminantium* (Fig. 3). IgM antibodies were first observed in small amounts 3 days after inoculation. Their concentration reached a maximum on the 4th day, after which these immunoglobulins became undetectable 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 concentration subsequently decreased.

Detection of antigen

The presence of *C. ruminantium* antigen in the blood fractions of heartwater-infected sheep was demonstrated by ELISA (Fig. 4). Plasma, the red blood cell and white blood cell fractions exhibited infected to non-infected ratios higher than 1,5. *C. ruminantium* antigen was first detected in plasma and serum on the 4th day after inoculation.

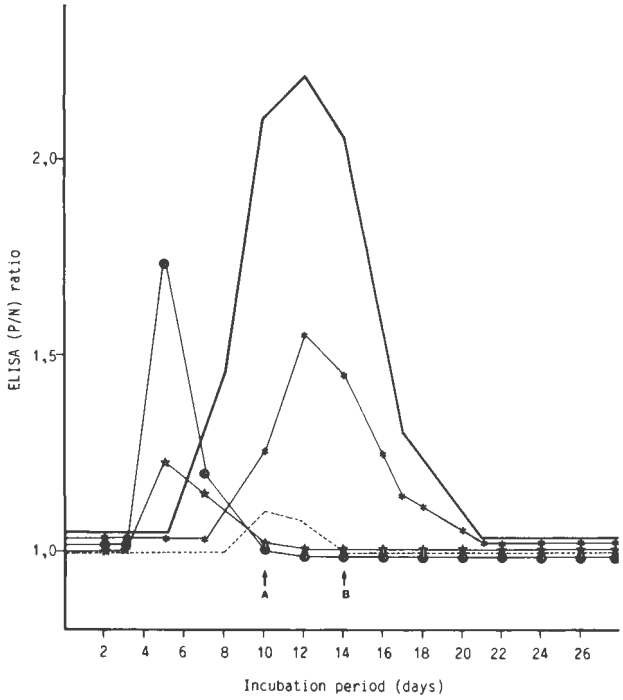

FIG. 4 ELISA screening of *Cowdria ruminantium* antigens in blood fractions of heartwater-infected sheep. (A) Day of reaction. (B) Day of treatment. Serum (★★); plasma (●●); whole blood (------); red blood cell fraction (———) and white blood cell fraction (**).

In the red 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, red blood cells 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 were only 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. This phenomenon could explain the increase of free IgM upon treatment of the disease with oxytetracycline, which inhibited proliferation of the organism. The antibody assay showed that IgM antibodies reached a maximum on the 4th day after infection and disappeared on the 7th day. IgG antibodies appeared on the 8th day and continued to increase up to at least the 28th day.

It appears 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. The organism is at first associated with the red blood fraction and subsequently with the white blood fraction. Concomitantly a decrease of *C. ruminantium* levels in plasma and serum occur. On Day 21, i.e. 7 days after treatment of the diseased animals, no *C. ruminantium* antigen 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 proteins. There is at present no evidence showing the organism to be either adsorbed to the cell surface or to be inside the cell. This is one reason why our cell fractions were not washed after the cell separation centrifugation step. This should be further investigated.

It should be remembered that the red blood cell fraction probably contains a large proportion of neutrophils since these cells have sedimentation coefficients and densities close to those of erythrocytes (Peeters, 1979). The *C. ruminantium* antigen detected in the red cell fraction may thus represent organisms taken up by neutrophils through phagocytosis (Wakelin, 1984). Some of these organisms may survive in these microbodies by mechanisms described for several intracellular parasites (Moulder, 1985) and are subsequently released and then enter endothelial cells in which they proliferate to form a colony (Cowdry, 1925; 1926).

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