Epidem. Inf. (1988), **100**, 205–212
Printed in Great Britain

Enzyme-linked immunosorbent assays for the measurement of specific antibodies in experimentally induced ovine toxoplasmosis

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(*Accepted 5 October 1987*)

SUMMARY

Tachyzoites of the RH strain of *Toxoplasma gondii* were inoculated intravenously into sheep following which serum samples were collected at approximately weekly intervals for 9 months. The sera were examined by the toxoplasma dye test and two enzyme-linked immunosorbent assays (ELISA) specifically developed for investigations of ovine toxoplasmosis. One was an antibody class capture assay for the detection of anti-toxoplasma specific IgM, the other an indirect assay which detected anti-toxoplasma IgG.

Some of the sheep had antibodies to toxoplasma prior to inoculation but none had specific IgM. Sera collected 17 days after inoculation showed that all had raised specific antibody levels but the only sheep that produced specific anti-toxoplasma IgM were those that were initially without any antibody. Specific IgM could be detected in all these particular sheep for at least 1 month after infection and up to 3 months in some. Specific IgG persisted at high levels for at least 3 months and could still be detected at moderate levels for at least 9 months.

The ELISA methods described are simple to perform and could clearly distinguish between previous infection and this experimental infection with *Toxoplasma gondii*.

INTRODUCTION

Recently, one of us (A.J.W.) carried out a study of *Chlamydia psittaci* (*ovis*) as a cause of abortion in sheep. In an attempt to eliminate toxoplasmosis as a possible complicating factor in this study it was decided to inoculate the ewes involved with tachyzoites of the RH strain of *Toxoplasma gondii* to ensure immunity in these animals before mating commenced. Blood samples were taken before inoculation and thereafter at approximately weekly intervals for 9 months. Aliquots of the serum samples thus obtained were made available to the Toxoplasma Reference Unit of the Swansea Public Health Laboratory (PHL) for antibody studies.

Antibody studies in experimentally induced ovine toxoplasmosis have been reported by Blewett, Bryson & Miller (1983). They investigated the possibility of using comparisons of the indirect haemagglutination and dye test titres, or the specific titres in the IgM and IgG antibody fractions as a means of distinguishing between acute and chronic infections. The methods used involved relatively large volumes of serum (1–2 ml) and as each specimen had to be eluted through a Sephadex G200 column, only small numbers could be handled at any one time. In an attempt to overcome these disadvantages it was decided to use enzyme-linked immunosorbent assays (ELISAs)

The serum samples were examined by the toxoplasma dye test (DT) (Fleck & Kwantes, 1980), an antibody class capture ELISA for toxoplasma-specific IgM antibody (IgM ELISA) and an indirect ELISA for toxoplasma-specific IgG (IgG ELISA).

This paper describes the above methods and demonstrates their value in measuring the immune response in sheep to an induced infection with *Toxoplasma gondii*.

MATERIALS AND METHODS

Specimens

Initially 60 ewes and 10 rams were used in this study. In order to judge the clinical response, one ewe and one ram were inoculated intravenously with 10^5 tachyzoites of the RH strain of *Toxoplasma gondii*. The following day blood samples were taken from all the ewes; the rams were bled a day later. These samples were required to establish the base-line immune status of the flock. After 3 days, when it had become obvious that the clinical response to the trial inoculation was not too severe, the other 59 ewes were inoculated with the same dose of tachyzoites. The remaining nine rams were not inoculated and serum samples from these were used as controls throughout. About a week later another 20 ewes were inoculated as described above.

Thereafter blood samples were taken from all the sheep at approximately weekly intervals for 9 months and the sera obtained from these samples were stored at −20 °C. When all the sera had been collected, aliquots of each were sent to the Swansea PHL where they were stored at −20 °C until tested.

Dye test (DT)

Sera were heated at 60 °C for 1 h to eliminate thermolabile anti-toxoplasma factors which may have been present and given rise to low-titre, false positive reactions in the dye test (Cathie, 1957).

After heating, serial twofold dilutions of the sera, in 25 μl volumes, were made in the wells of flat bottomed microtitre plates (Sterilin, Felton, England), and 50 μl of accessory factor serum added to each well. Accessory factor is comprised of fresh human serum tested to show that it contains sufficient complement activity and no toxoplasma antibody. A 25 μl volume of a suspension of tachyzoites of the RH strain of toxoplasma was then added and the microtitre plate incubated for 1 h at 37 °C. A 25 μl volume of an alkaline methylene blue

solution was added to each well and the microtitre plate incubated again for 10 min. The wells were examined with an inverted microscope and the titres recorded. Stained toxoplasma indicate absence of specific antibody whereas unstained toxoplasma indicate its presence.

IgM ELISA

Production of monoclonal antibody CIE3

This work was carried out at the Leeds PHL (unpublished data). Balb/c mice were each infected intraperitoneally with 10 cysts of the Gleadle strain of *Toxoplasma gondii* on days 1 and 28, followed by 100 trophozoites of the RH strain intravenously 3 days prior to fusion. Fusion was carried out with myeloma cells of the non-immunoglobulin secretory line designated P3-X63-Ag8.653 that was resistant to 8-azaguanine. Antibody production after fusion was monitored from day 10 by the toxoplasma dye test.

One hybridoma was found to produce an antibody (CIE3) with high specific activity against a 35 kDa surface-membrane antigen of the organism. After cloning, 10^6 cells were injected intraperitoneally into Balb/c mice previously primed with pristane (Sigma Ltd). When ascites was evident, it was tapped using a 14-gauge needle. The fluid was pooled, clarified by centrifugation and stored at −20 °C. When compared with the World Health Organisation anti-toxoplasma Standard Reference Serum (WHO International Laboratory for Biological Standards, Statens Seruminstitut, DK 2300, Copenhagen, S. Denmark), dye test titres equivalent to 32000 i.u./ml were obtained with this fluid.

Conjugation procedure

CIE3, an antibody of the IgG_3 isotype, was separated from the mouse ascitic fluid by affinity chromatography. A column measuring 10 × 1 cm containing Sepharose Protein A CL-4B (Pharmacia) was set up and equilibrated with 25 ml of 10 mM phosphate-buffered saline (PBS) pH 7·3. A total of 5 ml of the CIE3 mouse ascites was passed through and the column washed with PBS until the absorbance at 280nm was zero. The captured antibody was eluted with a 50 mM sodium acetate solution at pH 4·3 and dialysed overnight against 10 mM Na_2CO_3 solution. It was then conjugated to horseradish peroxidase (HRP) by the method of Wilson & Nakane (1978) and stored as small aliquots under liquid nitrogen.

Toxoplasma antigen

The antigen used throughout this study was produced from membranes of hens eggs infected with toxoplasma (Payne, Isaac & Francis, 1982). The chorioallantoic membranes of 13-day-old chick embryos were infected with a suspension of the RH strain of *T. gondii*. The trophozoites of the RH strain were obtained from the peritoneal cavities of mice infected 3 days previously. After 7 days incubation the membranes were removed, suspended in PBS and macerated. The suspension was then frozen at −70 °C and thawed at 37 °C on three occasions before being centrifuged at 2500 **g** for 10 min. The supernatant constituted the antigen and was stored at −20 °C.

Antigen and conjugate reagent

The approximate titre of both the antigen and the conjugate was determined by chessboard titration. The combined reagent was prepared by adding 100 μl of the conjugate to 100 ml of the antigen, mixing thoroughly, and incubating for 24 h at 37 °C. It was then divided into 5 ml volumes and stored at 4 °C. Several batches prepared over a 6 months period had working titres varying between 20 and 60 when titrated against standard sera.

Capture antibody

The optimum dilution of a rabbit anti-sheep IgM (anti-μ) (Nordic Immunology, Tilbery, The Netherlands) was determined by chessboard titration and found to be 1/1000 when used in the test conditions described. The 50 mM carbonate/bicarbonate coating buffer (pH 9·6) for the anti-sheep IgM was prepared by dissolving 1·56 g of Na_2CO_3 and 2·5 g of $NaHCO_3$ in 1 l of water. This could be used for up to 2 weeks if stored at 4 °C.

Substrate

The chromogenic substrate used was 3,3′, 5,5′, tetramethylbenzidene (TMB) (Miles Scientific, Naperville, USA). The stock solution was prepared by dissolving 100 mg TMB in 10 ml of dimethylsulphoxide. This was stored at room temperature in a dark bottle. Citric acid/sodium acetate buffer (100 mM) was prepared by dissolving 16·4 g of sodium acetate in 2 l of water and 2·1 g of citric acid in 100 ml of water. The citric acid solution was added to the sodium acetate solution to bring the pH to 6·0. This was then distributed in 100 ml volumes, autoclaved at 10 p.s.i./10 min and stored at room temperature. Just before use 250 μl of TMB solution was pipetted into a clean universal container and 25 ml of the pH 6·0 buffer added rapidly, with thorough mixing, followed by 25 μl of 6% (20 volumes) H_2O_2.

Wash solution and diluent

The wash solution consisted of PBS with 0·05% (v/v) Tween 20 (PBST) added. The diluent for both the serum specimens and antigen and conjugate reagent was PBST with the addition of 1% (w/v) bovine serum albumen (PBST-BSA).

Equipment

All the wash procedures were carried out with a Titertek Microplate Washer S8/12 and absorbance readings were made with a Titertek Multiskan MCC (Flow Laboratories, Rickmansworth, Herts, England).

IgM ELISA method

All tests were set up in flat-bottomed Falcon Microtest polyvinyl chloride flexible assay plates (Becton Dickenson, CA, USA). To 12 ml of coating buffer, 12 μl of anti-μ was added, mixed and distributed in 100 μl volumes to all wells of a microtitre plate. The plate was covered with a suitable lid and incubated for 1 h at 37 °C. After incubation the microtitre plate was washed three times. After removing any remaining wash solution by tapping the plate onto blotting paper,

1/100 dilutions of sheep sera were added in 100 μl volumes. After a further 1 h incubation at 37 °C, the plate was washed as previously described, 100 μl volumes of the antigen/conjugate reagent, diluted to titre, added to each well and the plate incubated for 1 h at 37 °C. The plate was washed and 100 μl of freshly prepared substrate added to each well. After incubation for 30 min at room temperature the reaction was stopped by adding 25 μl of 2M H_2SO_4 (Aristar) to each well and mixing gently but thoroughly, this changed the colour of the positive reactions from blue to yellow. The absorbances were read at 450 nm and recorded.

IgG ELISA method

An antigen consisting of cuticular material, obtained from tachyzoites of the RH strain of toxoplasma derived from peritoneal fluid of infected mice (Francis, Payne & Joynson 1987), was attached to wells of microtitre plates. After thorough washing to remove unattached antigen, 100 μl of a 1/100 dilution of each sheep serum under test was added to an appropriate, antigen-coated well. After incubation for 1 h at 37 °C the microtitre plate was washed and a 1/20000 dilution of a rabbit anti-sheep IgG conjugated to HRP (Nordic) added. Following incubation as previously described, the microtitre plate was washed again and 100 μl of TMB added to each well. The microtitre plate was then incubated and the reaction stopped, read and recorded as described for the IgM ELISA.

Because of some day-to-day variation in the OD readings, it was decided to record ELISA results as test (T) to negative (N) ratios (T:N ratios). The readings of the test samples were divided by the mean of readings obtained from the six negative samples from the rams at each sampling date:

$$\frac{\text{OD test sample}}{\text{Mean OD of 6 negative samples}} = \text{T:N ratio.}$$

A T:N ratio of 2 or more was considered to be a positive result.

RESULTS

The dye test results showed that prior to inoculation, of the 59 ewes examined in this study, 31 (53%) had toxoplasma antibody with titres ranging from 16 to 512 while 28 (47%) had no toxoplasma antibody (dye test < 16). Six of the nine rams had no toxoplasma antibody. The pre-inoculation samples from these sheep were also examined by the IgM and IgG ELISAs. The results (Table 1) show that the T:N ratios obtained with the IgG ELISA correspondingly increased with the dye test titres but specific IgM was not detected in any of the sheep.

Following inoculation, the antibody response of the 31 immune ewes differed from that of the 28 non-immune ewes.

The results summarized in Fig. 1 show that following inoculation the dye test and IgG ELISA gave closely related results with sequential specimens from the 31 immune ewes and that specific IgM could not be detected at any stage of infection. All the ewes showed a rise in antibody level and all had a dye test titre of at least 1024 at some stage of infection.

In contrast, specific IgM was demonstrated in the early stage of infection in all 28 non-immune ewes (Fig. 2). However, the T:N ratios obtained with the IgG

Table 1. *Toxoplasma antibody levels in the initial samples from 59 ewes and 9 rams*

No. of ewes	DT titre	IgG ELISA (T:N ratio)	IgM ELISA (T:N ratio)
28	< 16	1·0	1·0
3	16	3·0	1·0
3	32	3·7	1·0
9	64	4·8	1·0
5	128	5·0	1·0
7	256	5·3	1·0
4	512	5·5	1·0
No. of rams			
6	< 16	1·0	1·0
1	16	2·7	1·0
2	128	4·2	1·0

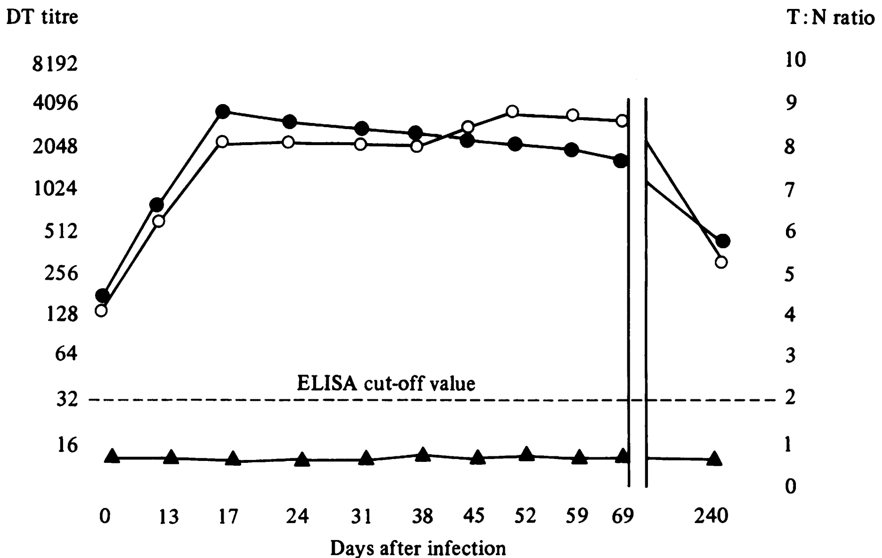

Fig. 1. Results obtained with sequential sera from 31 ewes with toxoplasma antibody prior to inoculation. (The geometric mean titres for each group of sera are shown). ●, Dye test; ○, Ig ELISA; ▲, IgM ELISA.

ELISA were lower when specific IgM was present. A close relationship of the IgG to the dye test was found only after specific IgM was no longer detectable.

Pre-inoculation samples from the 20 additional ewes brought into the experiment were not available. However, after inclusion in the study, these ewes were bled at the same times as the other sheep. The antibody response in 19 of these ewes was similar to that shown in Fig. 2 which suggests that they had no toxoplasma antibody prior to inoculation; the other ewe had an antibody response similar to that shown in Fig. 1.

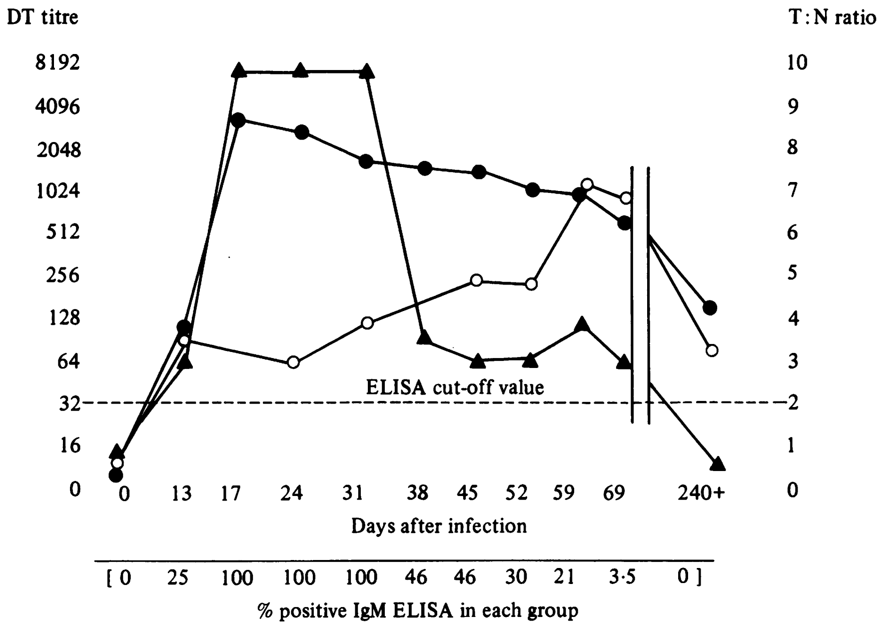

Fig. 2. Results obtained with sequential sera from 28 ewes without toxoplasma antibody prior to inoculation. (The geometric mean titres for each group of sera are shown and the percentages of sera giving a positive result in the IgM ELISA are shown below). ●, dye test; ○, Ig ELISA; ▲, IgM ELISA.

DISCUSSION

The results summarized in Figs. 1 and 2 suggest that the ELISA systems are detecting class-specific antibodies to *Toxoplasma gondii* whereas the dye test is detecting total specific antibody. Similar results were found when human sera were tested by anti-toxoplasma IgG and IgM ELISA systems and the results compared with the dye test (Francis, Payne & Joynson 1987). Balfour & Harford (1985) reported that negative anti-toxoplasma IgG ELISA results occurred with human sera which had high levels of anti-toxoplasma specific IgM and positive dye test titres. It is advisable, therefore, to test sera by both ELISA systems when acute infection is suspected.

With acquired toxoplasmosis in humans, specific IgM can usually be detected for about 6 months after onset of illness, by similar ELISA methods to those reported here. However, we have demonstrated that a worker who had acquired a laboratory infection with the RH strain produced specific IgM for about 3 months only (Payne *et al.* 1987). The relatively short duration of specific IgM found in these sheep may be due, therefore, to the mode of infection or the strain of toxoplasma used. The examination of specimens from sheep with acute, naturally acquired toxoplasma infection will be required to determine the length of time that specific IgM is normally present.

CIE3 antibody has been shown to recognize a major cell membrane component of *Toxoplasma gondii* against which IgM is produced in the course of human toxoplasmosis. It has also been shown to give a high level of protection in mice

against infection with this parasite (unpublished data). CIE3 antibody is also effective in an antibody-class capture assay for detecting human anti-toxoplasma specific IgM (Payne *et al.* 1987). It can be used at high dilutions with the potential of reducing both non-specific and background reactions, increasing specificity and sensitivity. The stability of this reagent is greatly improved when it is mixed with the toxoplasma antigen. No loss of titre could be detected in a conjugate-antigen mixture after storage for 9 months at 4 °C.

Many different antigen preparations were used during the development of this assay but the egg-derived antigen described, consistently gave the greatest distinction between positive and negative results.

The ELISA methods require only small volumes of serum (5 μl is sufficient for both assays) and large numbers of specimens can be tested with relative ease. We believe that the rapid, simple assays described above can clearly distinguish between past infection and acute experimental infection and should be capable of identifying naturally acquired cases of acute ovine toxoplasmosis.

A.J.W. acknowledges that the work at the Royal Veterinary College was supported by a grant from the Overseas Development Administration.

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