The development of antibody to *Cowdria ruminantium* in mice and its role in heartwater disease

**INTRODUCTION**

Heartwater disease has never been recognized to exist in mice as a result of natural infection. However a number of *Cowdria ruminantium* field strains of varying pathogenicity for the mouse have been discovered since 1971 when the Kümm strain (5) was first isolated. Pathogenicity of *C. ruminantium* infection in mice resembles that in ruminants and may vary with:

- the strain of *C. ruminantium* used;
- the strain of mouse used;
- the route of inoculation (6).

Although the development of antibody to *C. ruminantium* in various species of animals has been well documented in recent years (3, 6, 10) very little is known about its role in the immunity to heartwater. Experiments with transfer of serum of gamma globulins did not confer protection and *in vivo* neutralization tests have had varying results (4, 11). Many of these experiments were done before methods of determining antibody levels had been developed so it is not known whether any antibody was in fact present in the serum used for some of these experiments. However it was assumed that the protective immunity to heartwater was probably cellular and not humoral. To clarify the role of antibody in *C. ruminantium* infection the mouse model was used to investigate the development of antibody, and immune mouse and bovine sera were used to investigate the role of antibody in an *in vitro* neutralization assay.

**MATERIALS AND METHODS**

**Inoculation of mice and collection of sera**

To determine how antibody to *C. ruminantium* develops, groups of both Balb/c and DBA/2 mice were inoculated intravenously with 100, 10 and 1 LD50 of previously titrated mouse organ homogenate of Crystal Springs strain (2). Representative mice from each group were bled from the retro-orbital sinus at intervals of 2-7 days from day 7 after infection.

**Immune antisera**

**Mouse sera**

Hyperimmune mouse sera were obtained by immunizing mice according to the following schedule. Three inoculations were given at 28 day intervals:

- first inoculation : 100 LD50 intravenous (i/v) dose given intraperitoneally (i/p);
- second inoculation : 500 i/v LD50 doses given i/p;
- third inoculation : 100 i/v LD50 doses given i/v.

DBA/2 mice were inoculated with mouse organ homogenate, whereas Balb/c mice were inoculated with cell culture stable. Pooled serum from such mice with an IFAT titre of 1/2560 was used in the *in vitro* neutralization assays.
Bovine sera

Laboratory infection

Because the Crystal Springs strain of C. ruminantium is not highly pathogenic for bovines, animal No. 9.5 was splenectomized. Five weeks after splenectomy this animal was inoculated with 10 ml of passage 6 cell culture material of the Crystal Springs strain. Six days later it developed a temperature reaction which lasted 10 days and reached a maximum of 41.0 °C. C. ruminantium was isolated in cell culture from plasma (2) taken on days 1, 2, 3 and 7 of the temperature reaction and sub-inoculation of blood to sheep on days 1, 3 and 7 resulted in death of the sheep from heartwater (confirmed by brain smears). The serum used in the in vitro neutralization assays was taken 4 weeks post-inoculation and had an IFAT titre of 1/1280 and a western blot titre of 1/1000.

Field infection

This serum was a pool of equal amounts of serum taken from two bovines living on the Heartwater Project's field station in the Zimbabwe lowveld (a heartwater endemic area). These animals had developed increasingly high IFAT titres to C. ruminantium over a two year period. The serum used in the in vitro neutralization assay had an IFAT titre of 1/2500.

All sera were sterilized by filtration before carrying out the neutralization assay.

Indirect fluorescent antibody test

This was performed by the method of SEMU et al. (10). Antigen slides were made from bovine endothelial cells infected with the Crystal Springs strain of C. ruminantium. The reaction of the mouse sera was determined by anti-mouse polyvalent immunoglobulins FITC labelled antisera (Sigma) and that of the bovine sera by goat anti-bovine IgG FITC labelled antisera (Kirkegaard and Perry, Gaithersburg, MS, USA). Sera were tested at dilutions of 1/20 to 1/10240.

Cell culture stabilate

Four 900 cm² roller bottles (Costar, Van Nuys, CA, USA) with bovine aorta endothelial cells were infected with the Crystal Springs strain of C. ruminantium. When these bottles showed almost complete destruction of the monolayer (approximately ten days after inoculation), the culture supernatant was harvested into sterile centrifuge tubes and centrifuged at 30,000 g for 30 min at 4 °C. The supernate was discarded and the pellet was passed several times through a syringe with a 26 gauge needle to break up clumps of cellular material. The pellet was then made up to 50 ml in L15 medium as above, but containing 20 % NBCS, and cryopreserved in the presence of 10 % dimethyl sulphoxide (Sigma Chemical Co, St. Louis, MO, USA).

This infection stabilate was titrated out in 25 cm² flasks (Costar) of bovine endothelial cells to determine the LD₅₀ dose and was used at 100 LD₅₀ in neutralization assays.

In vitro neutralization assay

C. ruminantium cell culture stabilate was diluted in L15 medium to give 200 LD₅₀. Equal amounts (1.5 ml) of stabilate and immune serum were mixed and incubated at 4 °C for one hour. Five 25 cm² flasks of bovine endothelial cells were then inoculated with 0.5 ml of this mixture. Control cell cultures were inoculated with a similarly treated mixture of stabilate and normal serum. Flasks were incubated on a rocking platform at 37 °C for two hours to adsorb the inoculum, then the inoculum was poured off and the cell monolayers washed twice with 2 ml phosphate buffered saline (PBS) pH 7.4. Five ml of fresh L15 medium was added to each flask and cultures were then incubated at 37 °C on the rocker platform for up to 35 days. Flasks were examined daily for development of cytopathic effect (CPE). Smears were made at intervals from day 11 onwards, stained with Leukostat (Fischer scientific, Orangeburg, NY, USA) and examined for percent infection. Graphs were plotted (figures 3, 4, 5) representing the average percentage infection rate of five flasks.

RESULTS

In Zimbabwe the Crystal Springs strain was first isolated in 1988 (2). This strain was found to be pathogenic for both Balb/c and DBA/2 mice, with the i/p route requiring a larger dose of organisms than the i/v route to cause death. When infected i/v with the Crystal Springs strain of heartwater Balb/c mice died between 12-19 days post-inoculation; DBA/2 mice were more resistant, dying at 14-24 days post-inoculation.
Development of antibody in mice

Figures 1 and 2 show that the development of antibody to *C. ruminantium* in both DBA/2 and Balb/c mice began in the second week post infection and the development of peak titre was inoculation dose dependent.

Figure 1 shows that DBA/2 mice inoculated with 100 LD$_{50}$ dose achieved antibody titres of $1/160$ to $1/1280$ (average $1/618$) by day 14 post-infection. An inoculation dose of 10 LD$_{50}$ gave rise to lower antibody titres of $1/160$-$1/320$ (average $1/187$) by day 14, while 1 LD$_{50}$ gave antibody levels of between $0$-$1/320$ (1 mouse only) (average $1/20$). It was noticed that some DBA/2 mice survived a 10 and 100 LD$_{50}$ inoculation dose. Subsequently these mice were resistant to challenge with 100 LD$_{50}$. In contrast the DBA/2 mice inoculated with 1 LD$_{50}$ failed to develop high antibody titres and were susceptible to challenge even with the same dose (1 LD$_{50}$), except for the one mouse which developed an antibody titre of $1/320$ after the first inoculation.

Figure 2 shows that Balb/c mice inoculated with 100 LD$_{50}$ achieved antibody levels of $1/160$ to $1/640$ (average $1/480$) by day 18 post-infection. A dose of 10 LD$_{50}$ gave rise to antibody levels of $1/80$ to $1/320$ (average $1/187$) by the same day. These titres were less than those of the DBA/2 mice on day 14. All of these Balb/c mice died. Those inoculated with 1 LD$_{50}$ failed to develop any antibody and were fully susceptible to challenge.

**In vitro neutralization assays**

Immune serum having a high titre in the IFAT were used to perform an *in vitro* neutralization test in 25 cm$^2$ flasks of bovine endothelial cells.

![Reciprocal antibody titre](image1)

**Fig. 1**: Development of antibody to *C. ruminantium* in DBA/2 mice.

**Fig. 2**: Development of antibody to *C. ruminantium* in Balb/c mice.

Figure 3 shows that pooled immune serum from DBA/2 mice had the ability to almost completely neutralize 100 LD$_{50}$ *C. ruminantium* infection of bovine endothelial cells. In fact only one out of 5 flasks inoculated with DBA/2 immune serum/stabilate mixture became infected in this experiment compared with five out of five for the normal serum/stabilate control. This flask only began to show CPE on day 17 post-inoculation, whereas all flasks inoculated with normal serum/stabilate mixture were showing CPE by day 10 post-inoculation. Infection rates *in vitro* on day 14 post-infection averaged 10.5 % for normal serum/stabilate mixture but only 0.2 % for immune serum/stabilate mixture.

With pooled Balb/c serum (fig. 4) all flasks inoculated with both normal serum/stabilate mixture and immune serum/stabilate mixture became infected. However there...
was a difference in the infection rate, with the normal serum/stabilate controls showing a higher average infection rate compared with cultures inoculated with C. ruminantium inoculated with immune serum/stabilate.

Bovine sera (fig. 5) also showed a neutralizing effect. Only two out of five flasks inoculated with laboratory infected bovine serum/stabilate became infected and one out of five flasks inoculated with field bovine serum/stabilate became infected. There was also a delay in the first appearance of CPE in those flasks inoculated with immune serum/stabilate (day 14 for normal serum, day 21 for laboratory infected bovine serum, and day 25 for field infected bovine serum). Infection rates at day 21 post-inoculation averaged 17.6% for normal serum/stabilate, 0.5% for laboratory infected bovine serum/stabilate, and nil for field infected bovine serum/stabilate.

Fig. 4: In vitro neutralization. Balb/c mouse serum. (* IFAT titre 1/2560)

**DISCUSSION**

The experiments presented here have demonstrated that in mice antibody to *C. ruminantium* develops in the second week post-infection and that the antibody level is dependent upon the inoculation dose. In addition, hyperimmune serum from both DBA/2 and Balb/c mice contains antibodies which prevent either the adhesion of *C. ruminantium* to endothelial cells or the entry of *C. ruminantium* into endothelial cells in *vitro*. A similar effect was demonstrated for two bovine sera, one from a laboratory infection, one from field infection.

It is interesting to note that the strain of mouse which had greater resistance to heartwater (DBA/2) was able to produce a higher titre of antibody on initial infection (fig. 1) and also a more effective neutralization (fig. 3). Although immune serum from Balb/c mice had the same IFAT titre as immune serum from DBA/2 mice it was not nearly as efficient in neutralization. This indicates that neutralization ability of the immune sera differs in the two strains of mice and may also differ from the antibody reacting in the IFAT.

It should be also noted that serum from bovines infected in the field (and presumably constantly re-exposed to *C. ruminantium* infection) gave a greater neutralizing effect than serum from a laboratory infected bovine. However in this case there was also a corresponding difference in the IFAT.

The role of antibody in heartwater infections has been debated over a long period of time. Investigators have attempted passive transfer of antibody to susceptible animals, but were unable to confer protection either with serum or large quantities of gamma globulin from immune or hyperimmune animals, whether given simultaneously with the infection, during the incubation period or during the clinical reaction (reviewed by UILENBERG (11)). In this laboratory, experiments with DBA/2 and Balb/c mice have shown that transfer of immune serum or immune serum plus complement failed to protect either DBA/2 or Balb/c mice against an i/v challenge of *C. ruminantium* (data not shown).

Several attempts at in vitro neutralization have been made previously, with varying results (reviewed by UILENBERG (11)). In this laboratory we have been unable to demonstrate any neutralizing effect in DBA/2 or Balb/c mice inoculated i/v with a mixture (incubated at 4°C for one hour prior to inoculation) of immune serum and 100 LD50 of either percoll purified EBs (DBA/2 mice) or cell culture stabilate (Balb/c mice) (data not shown). A recent paper by DU PLESSIS (4) reported that incubation of hyperimmune sheep sera with a tick homogenate of the Kümm strain of *C. ruminantium*, inhibited the infectivity of the homogenate for outbred Swiss mice inoculated by the i/p route, but only if the incubation was carried out in the presence of complement. In the absence of complement the immune serum/tick homogenate was fully infective.

Fig. 5: In vitro neutralization. Bovine serum. (*IFAT titre 1/1280; # IFAT titre 1/2560)
The fact that neutralization is not obtained in vivo using sera which gives in vitro neutralization in the studies of the authors may be related to the possibility that the final antibody titre of the transferred sera is too low to cause effective neutralization or that the coating of C. ruminantium by immune antibody actually facilitates an establishment of infection in phagocytic cells e.g. neutrophils and macrophages. It is known that C. ruminantium can multiply in neutrophils in vivo and infect the peritoneal macrophages of mice (6). C. ruminantium EBs incubated with immune serum and then injected into animals by either the i/v or i/p route may be inhibited from entering endothelial cells but would probably be taken up by macrophages or neutrophils by opsonization. If these opsonized C. ruminantium EBs were able to multiply in these cells clinical infection would result.

Failure to demonstrate any effect of antibody in vivo may also be due to the possibility that C. ruminantium infection, once established, could spread directly from cell to cell, as is suspected with some species of Ehrlichia (9) and Rickettsia (12). If this is the case organisms would escape exposure to antibody.

The results presented here indicate that neutralizing antibody may play an effective role in the protective immunity to heartwater by blocking adhesion to or invasion of endothelial cells. With the imminent development of new vaccine body may play an effective role in the protective immunity to heartwater by blocking adhesion to or invasion of endothelial cells, as is suspected with some species of Ehrlichia (9) and Rickettsia (12). If this is the case organisms would escape exposure to antibody.

REFERENCES


ACKNOWLEDGEMENTS

This study was funded by the United States Agency for International Development (Cooperative Agreement No. AFR-0435-A-00-9084-00) and the Government of Zimbabwe. The authors gratefully acknowledge the assistance provided by Gillian SMITH.