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

Les réponses immunitaires contre *Cowdria ruminantium* ont été étudiées en utilisant des souris DBA/2 et Balb/c comme modèle. Les deux souches de souris ont été inoculées avec 1, 10 ou 100 DL$_{50}$ de *C. ruminantium* (stock Crystal Springs). Des anticorps contre *C. ruminantium* ont commencé à se développer dans la deuxième semaine après l'inoculation et le titre d'anticorps dépendait de la dose de *C. ruminantium* inoculée. Le rôle possible des anticorps sur la maladie a été recherché au moyen des tests de neutralisation *in vitro*, utilisant des sérums de souris et de bovins. Les résultats ont montré que les sérums hyperimmuns des souris DBA/2 et Balb/c étaient capables de neutraliser l'infection *in vitro*, celui des souris DBA/2 montrant l'effet neutralisant le plus fort. Deux sérums de bovins, l'un d'un animal infecté au laboratoire et l'autre provenant d'un mélange de sérums de deux animaux infectés naturellement, ont également montré un effet neutralisant.

Mots clés : Bovin - Souris - Cowdriose - *Cowdria ruminantium* - Sérum - Anticorps - Infection expérimentale - Réaction de neutralisation - Réponse immunitaire.

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 (8).

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

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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 LD$_{50}$ 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 LD$_{50}$ intravenous (i/v) dose given intraperitoneally (i/p) ;

- second inoculation : 500 i/v LD$_{50}$ doses given i/p ;

- third inoculation : 100 i/v LD$_{50}$ doses given i/v.

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

Revue Élev. Méd. vét. Pays trop., 1993, 46 (1-2) : 197-201

197

B. Byrom S.M. Mahan A.F. Barbet

Bovine sera

Laboratory infection

Because the Crystal Springs strain of *C. ruminantium* is not highly pathogenic for bovines, animal No. 95 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 1/2560.

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.

Propagation of *C. ruminantium*

C. ruminantium (Crystal Springs strain) was propagated in bovine aorta endothelial cells maintained on Leibovitz's L15 medium (Gibco BRL, Grand Island, N.Y. USA) as described previously (1). The L15 medium contained 10 % tryptose phosphate broth (Difco Laboratories, Detroit, MI, USA) ; 5 % New Born Calf Serum (NBCS) (ICN Flow, Bucks, UK) ; 100 units/ml penicillin G (CAPS, Harare, Zimbabwe) ; 2.5 µg/ml amphotericin B (Fungizone, Squibb Laboratories, Transvaal, South Africa) ; 0.292 µg/ml L-glutamine (Gibco/BRL) ; and 4.5 µg/ml D-glucose (Gibco/BRL).

Cell culture stabilate

Four 900 cm^2 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 mono-layer (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 cm^2 flasks (Costar) of bovine endothelial cells to determine the LD_{50} dose and was used at 100 LD_{50} in neutralization assays.

In vitro neutralization assay

C. ruminantium cell culture stabilate was diluted in L15 medium to give 200 LD_{50}. Equal amounts (1.5 ml) of stabilate and immune serum were mixed and incubated at 4 °C for one hour. Five 25 cm^2 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 per cent 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 cm² flasks of bovine endothelial cells.
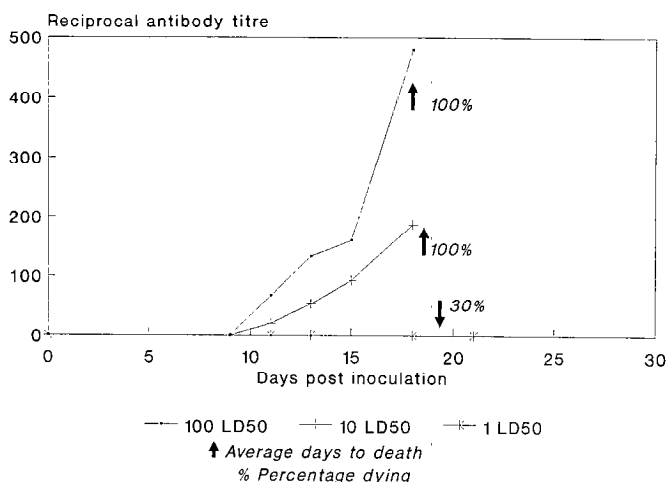
Fig. 2 : Development of antibody to C. ruminantium *in Balb/c mice.*

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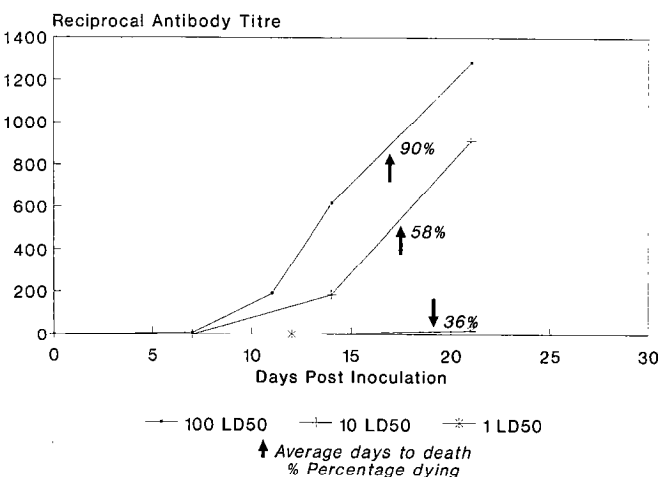
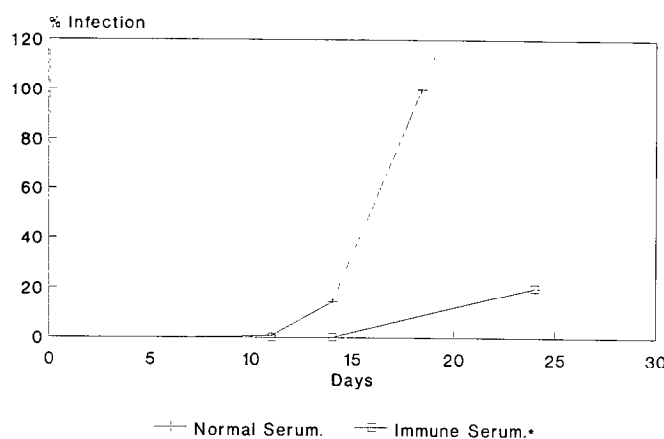*Fig. 1 : Development of antibody to* C. ruminantium *in DBA/2 mice.*

Fig. 3 : In vitro *neutralization. DBA/2 mouse serum. (* IFAT titre 1/2560)*
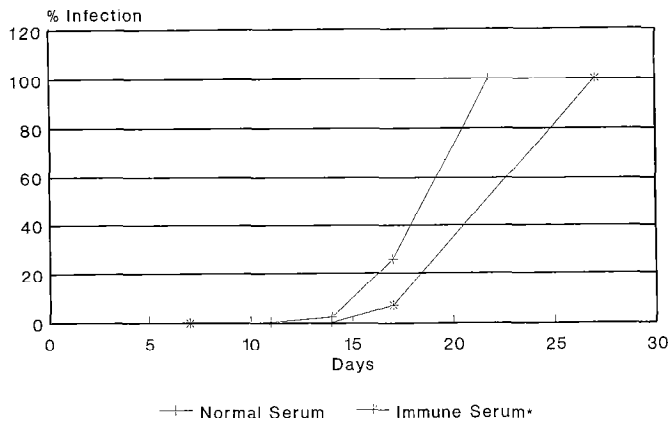
B. Byrom S.M. Mahan A.F. Barbet

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

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 fo 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.
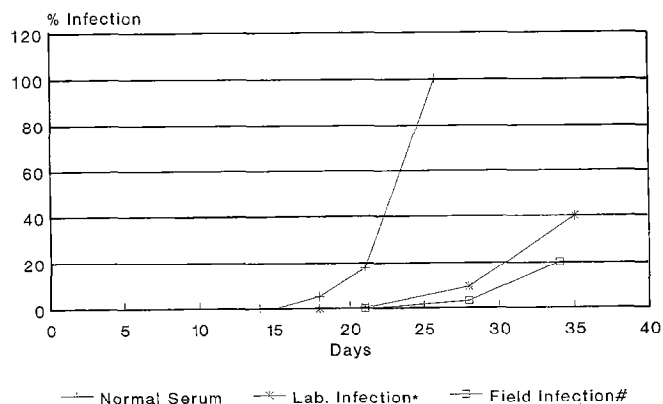

Fig. 5 : In vitro *neutralization. Bovine serum. (*IFAT titre 1/1280 ; # IFAT titre 1/2560)*

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 LD^{50} 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.

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* (7) 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 vaccines for heartwater, it could be important to determine which epitopes of the heartwater organisms react with neutralizing antibody and to include them in the vaccine.

ACKNOWLEDGEMENTS

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BYROM (B.), MAHAN (S.M.), BARBET (A.F.). The development of antibody to *Cowdria ruminantium* in mice and its role in heartwater disease .*Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 197-201

DBA/2 and Balb/c mice were used as a model to study the immune responses to *Cowdria ruminantium*. Both strains of mice were inoculated with 1, 10 or 100 LD_{50} of the Crystal Springs strain of *C. ruminantium*. Antibody to *C. ruminantium* started to develop in the second week post-inoculation and the titre of the antibody was dependent on the inoculation dose of *C. ruminantium*. The possible role of antibody in heartwater disease was studied by *in vitro* neutralization assays using both mouse sera and bovine sera. Results of these tests show that hyperimmune serum from both DBA/2 and Balb/c mice had the ability to neutralize infection *in vitro*, with the DBA/2 serum showing a greater neutralizing effect. Two bovine sera, one from a laboratory infected animal and one a pool from two animals infected in the field also gave a neutralizing effect.

Key words : Cattle - Mice - Heartwater - *Cowdria ruminantium* - Sera - Antibody - Experimental infection - Neutralization test - Immune response.

BYROM (B.), MAHAN (S.M.), BARBET (A.F.). Aparición de anticuerpos a *Cowdria ruminantium* en roedores y su importancia para la cowdriosis.*Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 197-201

Con el fin de estudiar la respuesta inmune a *Cowdria ruminantium*, se inocularon ratones DBA/2 y Balb/c a 1, 10 o 100 DL_{50}, de la cepa "Crystal Springs" de *C. ruminantium*. Los anticuerpos a *C. ruminantium* aparecieron dos semanas post inoculación, con títulos que variaron de acuerdo a las dosis de inoculación de *C. ruminantium*. El posible papel de los anticuerpos en la cowdriosis, se estudió mediante seroneutralización *in vitro*, tanto con los sueros de ambos tipos de ratón, como con sueros bovinos. Los resultados de estas pruebas muestran que los sueros hiperinmunes de los ratones, tanto DBA/2 como Balb/c, lograron neutralizar la infección *in vitro*, observandose un mayor efecto neutralizante de los DBA/2. El efecto neutralizante se observó también con dos sueros bovinos, uno proveniente de un animal de laboratorio infectado y el otro de un "pool" obtenido a partir de dos animales infectados bajo condiciones de campo.

Palabras claves : Bovino - Ratón - Cowdriosis - *Cowdria ruminantium* - Suero - Anticuerpo - Infección experimental - Reacción de neutralización - Repuesta inmunológica.