

An *in vitro* test to demonstrate the inhibitory effect of homologous immune serum on the infectivity of *Cowdria ruminantium*

J. L. DU PLESSIS

Onderstepoort Veterinary Institute, Onderstepoort, 0110 South Africa

ABSTRACT

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Homologous immune sheep serum inhibits the infectivity of mice for the Kümm stock of *Cowdria ruminantium* in an *in vitro* test. The presence of complement and an optimal ratio between the reagents are of cardinal importance. With the exception of the Ball 3 stock, there is no cross-inhibition between the immune sera of sheep infected with several other heterologous stocks and the Kümm stock. The role played by the humoral component of the immune response in naturally acquired heartwater is unknown, but probably small compared to the cellular component.

INTRODUCTION

The immunity in heartwater is considered to be cell-mediated rather than humoral (Stewart 1987). The view that antibodies do not influence the course of the infection was largely based on the finding that serum or large quantities of gamma globulin from immune animals fail to influence the outcome of the disease, irrespective of whether it is given at the time of infection or during the incubation period (Alexander 1931; Du Plessis 1970 & 1982).

Evidence has recently been obtained that immune serum inhibits the infectivity of the heartwater agent in the presence of complement and mouse peritoneal macrophages, but that this inhibitory effect cannot be demonstrated in the absence of macrophages (Du Plessis, Berche & Van Gas 1991).

Since it is important to know whether antibodies play a role in the heartwater immune response and since no explanation was given why macrophages

appear to be essential, it was decided to do further experiments in which optimal quantities of the reagents would first be determined.

MATERIALS AND METHODS

Cowdria ruminantium infective inoculum

Cowdria ruminantium-infected tick homogenate, also used in earlier experiments (Du Plessis *et al.* 1991), was prepared as described (Du Plessis 1982) from 100 engorged *Amblyomma hebraeum* nymphae that had fed on a sheep infected with the Kümm stock. The ticks were homogenized in 40 ml buffered lactose peptone (BLP), centrifuged at 1 000 r.p.m. for 5 min and the supernatant deep-frozen in liquid nitrogen in suitable quantities. A sample was withdrawn and an infectivity titre of $10^{4.8}$ determined in mice, according to the procedure previously described (Du Plessis 1982).

Mice

Female, conventional, outbred Swiss white mice, 6–8 weeks old, were used in all the experiments.

Preliminary experiments

Stabilizing effect of bovine foetal serum

In an earlier study (Du Plessis 1982), in which it was found that both pre-infection and convalescent sheep serum had an inhibitory effect on the infectivity of the heartwater agent, the addition of bovine foetal serum (BFS) enabled an incubation time of 2 h at 37 °C without excessive loss of infectivity. To ascertain whether BFS was also required as a stabilizing agent in the present series of experiments, 2 10-fold serial dilutions of the Kümm stock infective inoculum (KSII) were prepared in BLP. One ml of the 10⁻² dilution was transferred to each of 4 test tubes. 0.5 ml BFS was added to 2 of these and an equal volume of BLP to the other 2. One tube with BFS and 1 without were incubated at 37 °C for 1 h and the other 2 for 30 min. Four groups of 5 mice were then inoculated intraperitoneally (i.p.) with 0.3 ml of the 4 samples. A further 4 groups of 5 mice were inoculated with the 10⁻³ dilution of the KSII with and without BFS. Mouse mortalities were recorded and the specificity of the mortalities confirmed as having been due to *C. ruminantium* infection as previously described (Du Plessis & Malan 1988). Serum samples obtained 4 weeks after inoculation from mice that had survived were subjected to the indirect fluorescent antibody (IFA) test conducted as previously described (Du Plessis & Malan 1987).

Concentration of complement

Since it has been shown that the infectivity of *C. ruminantium* is adversely affected by complement (C') (Du Plessis, Malan & Kowalski 1987) and that the infectivity is inhibited by an immune serum in the presence—but not in the absence—of rabbit C' (Du Plessis *et al.* 1991), an experiment was carried out to determine the highest dilution of the KSII that would retain its infectivity in the presence of the highest possible concentration of C'. This would ensure that the mice would not be infected with overwhelming numbers of *Cowdria* in the final *in vitro* test in which the heartwater agent would be exposed to an immune serum and C'.

To 1 ml of a 10⁻², 10⁻³ and 10⁻⁴ dilution of KSII was added 0.1 ml of reconstituted freeze-dried rabbit serum (the source of C')* and 0.4 ml BFS. Likewise 0.2 ml rabbit serum was added to similar volumes of KSII and 0.3 ml BFS. After incubation of the 6 samples at 37 °C for 30 min, 5 mice per sample were inoculated i.p. with 0.3 ml per mouse.

Immune sera and *C. ruminantium* infectivity

It was decided to use 0.2 ml undiluted rabbit serum per 5 mice and a 10⁻³ dilution of KSII to test the

effect of immune serum on *C. ruminantium* infectivity. The sera of 3 sheep infected with the Kümm stock, collected 2–3 weeks after the height of the febrile reaction, was used in 3 separate tests. The sheep had been treated with oxytetracycline on the 3rd day of the febrile reaction. In each of these tests a volume of 0.2 ml undiluted immune serum, 0.6 ml BFS (0.8 ml in the absence of C') and 1 ml of a 10⁻³ dilution of KSII were incubated in the presence of 0.2 ml rabbit serum at 37 °C for 30 min. The immune serum without C' and the serum of a sheep susceptible to heartwater, with and without C', were incubated in the presence of KSII and BFS as controls. Four groups of 5 mice each were inoculated i.p. with 0.4 ml per mouse.

In a final experiment, the sera of sheep infected with heterologous stocks of *C. ruminantium* were compared with the homologous serum used in the previous experiment. Antisera to the Ball 3, Welgevonden, Kwanyanga and Mali stocks were obtained from sheep inoculated with deep-frozen sheep blood infected with the above stocks as previously described (Du Plessis, Van Gas, Olivier & Bezuidenhout 1989). The sheep were treated with oxytetracycline on the 3rd day of the febrile reaction and bled 4 weeks after infection. The sera were subjected to the IFA test and stored at -18 °C. A non-immune serum was included as control. The same volumes of serum, C', BFS and KSII were used as described in the previous experiment.

RESULTS

Mouse mortalities

Once mice commenced showing clinical signs of a ruffled hair coat, listlessness and dyspnoea 12–15 d after having been inoculated, they invariably died. The sera of those that survived with few exceptions, were negative in the IFA test at a dilution of 1:20. Since antibodies are produced only if infection is established and replication of *Cowdria* takes place (Du Plessis & Malan 1987), it can be concluded that the viable infective organisms in the inocula had been reduced to numbers too small to initiate infection in the case of mice that survived.

Preliminary tests

It can be seen from Table 1 that a prolonged incubation period of 1 h adversely affected the infectivity of both the 10⁻² and the 10⁻³ KSII dilutions. The stabilizing effect of BFS becomes evident in this period of incubation. If one compares Groups 5 and 7 on one hand and Groups 6 and 8 on the other, it can be seen that there was a decreased mortality rate in the absence of BFS and therefore poorer preservation of infectivity. In order to be able to use the 10⁻³ KSII dilution in future experiments and thereby avoid an overwhelming concentration of

* South African Institute for Medical Research

TABLE 1 The effect of bovine foetal serum on the infectivity of the heartwater agent *in vitro*

Group	Incubation time (min)		Bovine foetal serum	<i>C. ruminantium</i> concentration		Mouse mortality
	30	60		10 ⁻²	10 ⁻³	
	1	+			+	
2	+		+	+	5	
3	+			+	4	
4	+			+	4	
5		+	+	+	4	
6		+	+	+	3	
7		+		+	2	
8		+		+	0	

TABLE 2 The effect of complement on the infectivity of varying concentrations of *C. ruminantium*

Group	Complement ml/5 mice		Dilution of Kümm stock inoculum			Mouse mortality
	0,1	0,2	10-2	10-3	10-4	
	1	+		+		
2	+			+		5
3	+				+	2
4		+	+			5
5		+		+		5
6		+			+	0

Cowdria, it was decided to place the immune sera in contact with the heartwater agent for 30 min in the presence of BFS.

The results summarized in Table 2 indicated that a KSII dilution of 10⁻³ and the addition of 0,2 ml rabbit serum per 5 mice would ensure an adequate amount of C' and avoid an unnecessarily high concentration of the heartwater agent and also maintain *Cowdria* infectivity. The detrimental effect of C' is shown by the fact that at the lower concentration of C', 2 out of 5 mice inoculated with the 10⁻⁴ KSII dilution died, while infectivity was totally destroyed at the higher concentration of C'.

Immune sera and *C. ruminantium* infectivity

It can be seen from Table 3 that in 3 separate experiments the infectivity of the heartwater agent was markedly inhibited by the sera of 3 different sheep that had recovered from infection with the Kümm stock of *C. ruminantium*. In Exps 1 and 2 only 2 and 1 mice, respectively, died, while in Exp. 3 not a single mortality was recorded. Except for Exp. 2 in which 4 out of 5 mice died, the infectivity of *Cowdria* was not affected by the same immune serum in the absence of C' (Table 3, Group 2). The essential role played by C' is therefore evident. The inhibitory effect can be attributed to the immune sera that contained high levels of antibody, as shown by IFA titres of 1:5120 and 1:20480, and not to an excess of C' or any other factor, since in Groups 3 and 4 the infectivity of *C. ruminantium*

TABLE 3 The *in vitro* effect of immune serum and complement on the infectivity of the Kümm stock

Group	Immune serum	Non-immune serum	Complement	Mouse mortality		
				Exp. 1	Exp. 2	Exp. 3
1	+			2(20480)*	1(5120)	0(20480)
2	+		+	5	4	5
3		+	+	5	5	5
4		+		5	5	5

*(20480)=reciprocal of IFA test titre

TABLE 4 The effect on the infectivity of the Kümm stock of antibodies against several heterologous stocks of *C. ruminantium*

Group	Immune serum		Mouse mortality
	<i>C. ruminantium</i> stock	IFA test titre	
1	Kümm	1:20480	0
2	Mali	1:20480	5
3	Welgevonden	1:1280	5
4	Kwanyanga	1:1280	4
5	Ball 3	1:1280	1
6	Non-immune control serum	—	5

incubated with a control serum was unaltered, irrespective of whether C' was added or not.

Except for the Ball 3 antiserum, the convalescent sera of sheep that had recovered from infection with the Mali, Welgevonden and Kwanyanga stocks had no inhibitory effect on the infectivity of the heterologous Kümm stock, whereas no mouse mortalities were recorded in the same experiment in the case of the homologous Kümm immune serum (Table 4). The Ball 3 convalescent serum, however, clearly reduced the infectivity of the Kümm stock, since only 1 out of 5 mice died. Although the antibody levels of the Welgevonden and Kwanyanga sera were well below that of the Kümm serum, the Mali serum that showed no inhibitory effect had a high IFA titre, while that of the Ball 3 serum was only 1:1280. The levels of antibody therefore did not seem to play an important role. In this experiment the maintenance of the KSII infectivity was proved by the mortality rate of 5 out of 5 mice inoculated with the control non-immune serum, C' and *Cowdria* (Table 4, Group 6).

DISCUSSION

The view hitherto held that immunity in heartwater is cellular rather than humoral, is mainly based on the finding that a specific subset of lymphocytes, the Lyt-2⁺ T cells, play a key role in the immunity of mice to the Welgevonden stock of *C. ruminantium*. The inability of athymic nude mice to mount an immune response against *Cowdria* was considered as sup-

portive evidence. Several findings also suggested that antibodies do not play a protective role. The inability of hyperimmune serum to influence the outcome of the disease (Alexander 1931; Du Plessis 1979, 1982; Ramisse 1971 cited by Uilenberg 1983) and the fact that calves born from immunized cows with demonstrable colostral antibodies were no more resistant to challenge than calves without antibody (Camus 1987), suggest that antibodies do not afford protection.

Furthermore, immune sheep with high titres of antibody are partially or fully susceptible not only to heterologous challenge (Du Plessis 1982; Du Plessis & Van Gas 1989; Du Plessis *et al.* 1989), but also to homologous challenge (Du Plessis *et al.* 1989). The finding in a recent study (Du Plessis, Looock & Lüdemann 1992) that no antibody was detectable with the IFA test in the serum of 42 cattle that were fully resistant to challenge and an earlier observation that a considerable number of serologically negative cattle were immune to challenge 6–24 months after experimental immunization (Du Plessis, Bezuidenhout & Lüdemann 1984), also suggest that resistance to challenge is not dependent on serum antibodies. In cattle there is therefore poor correlation between resistance to challenge and levels of antibody detectable with the IFA test. It must be pointed out though, that antibody is usually detectable in sheep (Du Plessis *et al.* 1989) and mice (Du Plessis *et al.* 1991) that are resistant to challenge.

This report records an *in vitro* serum-*Cowdria*-complement test system to assay the influence of antibodies on the infectivity of the heartwater agent. The finding that homologous immune sheep serum inhibits the infectivity of the Kümm stock under specific experimental conditions, necessitates a reconsideration of the protective role possibly played by serum antibodies. The present observation differs from an earlier finding that the inhibitory effect of immune serum was dependent on the presence of macrophages of naive mice (Du Plessis *et al.* 1991). The present experiments differed from the earlier test in that a 10-fold higher dilution of the Kümm stock infective inoculum and double the concentration of C' was used in the former. It would seem that a rather delicate balance between a minimal number of *Cowdria* and the maximal amount of C', that would not *per se* compromise the infectivity of the heartwater agent, has to be maintained in order to demonstrate the inhibitory effect of antibody-rich serum.

It would therefore appear that inhibition of *Cowdria* infectivity by immune sheep serum can be attributed either to opsonic antibodies or to a direct lytic effect on the heartwater agent through the antibody/C'-dependent pathway. The fact that a control serum, from a heartwater susceptible sheep,

failed to bring about the inhibitory effect indicates that specific antibodies are responsible for this effect. Although the indispensability of C' suggests that immune serum has a direct cowdricidal rather than an opsonic effect, additional experiments are required to clarify this point. The inhibitory effect of antibodies was demonstrable in the absence of macrophages during the incubation of the reagents, but phagocytosis and inactivation of *Cowdria* by the peritoneal macrophages of the mice injected with the inoculum may have been facilitated by opsonic antibodies. Although the immunity to challenge of cattle, in which antibodies are no longer detectable, would indicate that serum antibodies, whether through opsonic or cowdricidal activity, are not essential, it must be borne in mind that a negative IFA test does not imply the total absence of antibody and that only trace amounts of antibody may be required to promote protection.

Even though the extent to which serum antibodies participate in the development of protective immunity would appear to be small at this stage, the findings in this study nevertheless suggest that the immunity in heartwater is not solely cell-mediated, but that there is also a humoral component. This would be compatible with the finding that maximal immunity to the intracellular growth of the closely related *Ehrlichia canis* required the interaction of *E. canis* with both humoral and cellular factors in proper sequence (Lewis & Ristic 1978). It is also consistent with the protective synergism between cellular and humoral elements in the immunity of other obligate intracellular rickettsial agents such as *Rickettsia mooseri* (Gambrill & Wissemann 1973) and *Coxiella burnetii* (Kishimoto & Walker 1976).

The relative degree to which the cellular and humoral components determine immunity in heartwater requires further investigation. Whereas immune macrophages alone (Du Plessis *et al.* 1991) and even in the absence of C' (Du Plessis 1982) can protect mice against the Kümm stock, it cannot, as explained above, be concluded from the results obtained with the *in vitro* assay that antibodies alone afford protection. Furthermore, the question is justified whether observations made in mice infected with the Kümm stock can without reservation be extrapolated to domestic ruminants and other stocks of *C. ruminantium*.

It is noteworthy that in the *in vitro* test there was no cross-inhibition between the Kümm stock and antisera against the Welgevonden, Mali and Kwanyanga stocks. In cross-immunity tests there was also no cross-protection between sheep immune to the Kümm and these 3 stocks (Du Plessis *et al.* 1989; Logan, Birnie & Mebus 1987). The inhibitory effect of an anti-Ball 3 serum on Kümm stock demonstrated in the present study was, however, unexpected, because none of 10 Ball 3-immune

sheep were resistant to challenge with the Kümm stock in a previous experiment (Du Plessis *et al.* 1989).

Previously (Du Plessis *et al.* 1991) anti-Ball 3 and anti-Welgevonden serum failed to inhibit the infectivity of the Kümm stock, even though the IFA test titre of the anti-Ball 3 serum was 4 × higher than that of the serum used in the present study. The homologous antiserum in the same experiment, however, also had no effect. The only differences between the 2 experiments are the lower concentration of the heartwater agent and the larger amount of C' in the 2nd attempt, underlining once more the importance of an optimal ratio between the reagents in the *in vitro* test.

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